JYU DISSERTATIONS 465

Riikka Kuosmanen

The Effect of Structure on the Gel Formation Ability and the Properties of Bile Acid Based Supramolecular Organogels

UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
AND SCIENCE

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ABSTRACT

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The thread behind this thesis has been fascinating. The study has focused on how changes in the alkyl side chain structure of the bile acid derivatives effects on the self-assembly properties of the compounds. Side chain structures were chosen not to contain any functional groups because of the novelty of the compounds in the field of steroidal supramolecular gels. In addition, the work could provide important insights on how to design supramolecular gelator molecules with divergent properties.

The 19 compounds presented in this thesis work were prepared by mixed anhydride synthesis method. All the compounds were characterized with NMR-spectroscopy and mass spectrometry. Single crystal structures were obtained from majority of the compounds studied. Additionally, some of the compounds were investigated by solid state NMR. The gelation abilities of the compounds were observed by utilizing conventional test-tube inversion method in divergent solvents and, in a few cases, aqueous solvent mixtures. Morphology of the dried gel systems were studied by scanning electron microscopy.

In this thesis, the structure of the side chain and the nature of the bile acid backbone were observed to affect the self-assembly properties of the compounds. For example, the side chain had a major impact on the stability of the gels when the gels were exposed to physical stimuli (disturbed with a spatula). SEM-images revealed that the bile acid backbone impacted on the morphology of the gel system as fibrous or spherical structures were seen depending on the amount of hydroxyl groups in the bile acid backbone.

As a conclusion, this work provides a perspective on how the gelator structure effects on the self-assembly ability of the compound and properties of the formed gel systems.

In addition to this thesis, a review article of steroidal supramolecular metallogels was published (*Chem. Soc. Rev.*, **2020**, *49*, 1977–1998) during the making of the current thesis work.

Keywords: Supramolecular chemistry, Supramolecular gels, Bile acids, Steroids

TIIVISTELMÄ

Kuosmanen, Riikka
Rakenteen vaikutus sappihappopohjaisten supramolekulaaristen geelien muodostumiseen ja muodostuneiden geelien ominaisuuksiin
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Tämän väitöskirjatutkimuksen takana oleva punainen lanka on ollut hyvin kiehtova. Tutkimus on keskittynyt siihen, millä tavalla tutkittujen sappihappojohdannaisten sivuketjun pituus ja muoto vaikuttaa kyseisten yhdisteiden itsejärjestäytymisominaisuuksiin. Tutkittaviksi valitut sivuketjut eivät sisältäneet funktionaalisia ryhmiä, jotta saatiin aikaan uusia steroidipohjaisia supramolekulaarisia gelaattoreita. Lisäksi väitöskirjatutkimus tarjoaa uusia näkökulmia haluttuja ominaisuuksia omaavien supramolekulaaristen gelaattoreiden suunnitteluun.

Tässä työssä esiteltävät 19 yhdistettä valmistettiin seka-anhydridimenetelmän avulla. Kaikki yhdisteet karakterisoitiin NMR-spektroskopian ja massaspektrometrian avulla. Suurimmasta osasta yhdisteitä määritettiin myös yksikiderakenteet. Osaa yhdisteistä tutkittiin myös kiinteän tilan NMR-spektroskopian avulla. Kaikkien tutkittujen yhdisteiden geelinmuodostusominaisuuksia tutkittiin koeputken kääntömenetelmällä eri liuottimissa. Muutamien yhdisteiden itsejärjestäytymistä tutkittiin myös erilaisissa vesiliuoksissa. Pyyhkäisyelektronimikroskopiaa (SEM) hyödynnettiin tarkasteltaessa kuivattujen geelien rakenteita.

Tutkittujen yhdisteiden kohdalla havaittiin sekä sivuketjun että sappihapon rakenteen vaikuttavan yhdisteiden itsejärjestäytymisominaisuuksiin. Esimerkiksi sivuketjun pituudella oli merkittävä vaikutus syntyneiden geelien mekaaniselle stabiilisuudelle, kun niitä häirittiin spaattelin avulla. Yhdisteen rungon, eli sappihappo-osan, havaittiin olevan merkittävässä roolissa muodostuneiden geeliverkostojen rakenteeseen: riippuen sappihapporungon sisältämien hydroksyyliryhmien lukumäärästä nähtiin joko kuitumaisia tai pallomaisia rakenteita. Näiden havaintojen johdosta tämä väitöskirja tarjoaa välineitä sen arviointiin, miten gelaattorin rakenne voi vaikuttaa geelinmuodostuskykyyn ja muodostuneiden geelien ominaisuuksiin.

Väitöskirjatyöskentely on tuottanut tämän väitöskirjan lisäksi alan arvostetussa lehdessä julkaistun katsausartikkelin (*Chem. Soc. Rev.*, **2020**, *49*, 1977–1998).

Avainsanat: Supramolekyylikemia, Supramolekulaariset geelit, Sappihapot, Steroidit

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PREFACE

This thesis work was carried out at the Department of Chemistry, University of Jyväskylä, Finland, between 2015 and 2021. The work was funded by Ellen and Artturi Nyyssönen Foundation and the University of Jyväskylä Graduate School for Doctoral Studies. Additional travel grants were received from Gust. Komppa Foundation and the Department of Chemistry. For making the thesis work financially possible, all the mentioned instances are gratefully acknowledged.

For exceptionally inspiring guidance and supervision, Professor Kari Rissanen and University lecturer, Docent Elina Sievänen are deeply thanked. You have given me such outstanding support during these years, especially during both of my pregnancies and when I returned to continue thesis work from blissful "baby bubbles". And during the gelation process of this thesis! I have learned immensely from you two.

The staff of the department is thanked for making this place so wonderful to work in, and for all the help I have received during this project. Some of you have become good friends of mine. I am also deeply grateful for all the students I have had the pleasure to guide in the lab.

The group K! Thank you all, past and present, for welcoming me into the group warmly. You have cultivated me from a very, very introvert person to quite talkative chatterbox.

Although this project has been both fascinating and demanding, it would have been much more challenging without my dear friends and family. Special thanks (and apologies) go to Elina L., Susanna, Tiina, Anniina, and Lila for listening and taking my mind of the thesis work. My parents-in-law, Eva and Pekka, and sisters-in-law, Anu, Leena and Anna: you all have been great help and support during these years. Thank you all especially for taking care of our children whenever help was needed. Next, my family. Parents Terttu and Antero, thank you for everything. You have always been a great support and encouragement whenever needed, whether the matter was studies, work, or babies. Riina, dear sister, thank you for being there always! Love of my life, Aleksi, I am so grateful for all the love and support you have given me over the past eleven years (I must have been extremely annoying lately, I'm sorry). We have had two absolutely wonderful daughters, Sofia and Hilda, both synthetized during this thesis work. The two little princesses have been the best distraction from work.

Jyväskylä 18.11.2021
Riikka Kuosmanen

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the original publications listed below, which in text are referred to by their Roman numerals.

I Riikka Kuosmanen, Rakesh Puttreddy, Roosa-Maria Willman, Ilkka Äijäläinen, Adéla Galandáková, Jitka Ulrichová, Hannu Salo, Kari Rissanen and Elina Sievänen, Biocompatible hydrogelators based on bile acid ethyl amides, *Steroids*, **2016**, *108*, 7-16.

II Riikka Kuosmanen, Rakesh Puttreddy, Kari Rissanen and Elina Sievänen, Systematic modulation of the supramolecular gelation properties of bile acid alkyl amides, *Chem. Eur. J.*, **2018**, *24*, 18676-18681.

III Riikka Kuosmanen, Khai-Nghi Truong, Kari Rissanen and Elina Sievänen, The Effect of the Side Chain on Gelation Properties of Bile Acid Alkyl Amides. *ChemistryOpen*, **2021**, *10*, 1150–1157.

Author's contribution

The author is responsible for the synthesis and gelation tests of all compounds. She is also responsible of the NMR spectroscopic characterization of the compounds and writing the manuscripts, excluding the X-ray crystallographic, ^{13}C CPMAS NMR, and biological studies sections. In the case of publication II, she performed the T_{gel}-measurements.

ABBREVIATIONS

AFM	atomic force microscopy
ACN	acetonitrile
CdS	cadmium sulfide
cryo-SEM	cryogenic scanning electron microscopy
cryo-TEM	cryogenic transmission electron microscopy
$CuCl_2 \cdot 2H_2O$	copper (II) chloride dihydrate
$CuSO_4 \cdot 5H2O$	copper (II) sulfate pentahydrate
$Cu(NO_3)_2 \cdot 3H_2O$	copper (II) nitrate trihydrate
DBS-$CONHNH_2$	1,3:2,4-dibenzylsorbitol-$CONHNH_2$
DCM	dichloromethane
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
EDTA	ethylenediamine tetraacetic acid
LiCl	lithium chloride
LMWG	low molecular weight gelator
MBS-CO_2Me	1:4-aldehyde:sorbitol-CO_2Me
MeOH	methanol
NaBr	sodium bromide
NaCl	sodium chloride
SEM	scanning electron microscopy
TEM	transmission electron microscopy
THF	tetrahydrofuran
w/v	weight to volume ratio

CONTENTS

1 REVIEW OF THE LITERATURE

1.1 Supramolecular gels

Supramolecular gels are a relatively young, yet ample, branch of science. Numerous different types of molecules,[1–5] or mixtures of them,[6] have been discovered to act as gelators (Figure 1): urea- and sugar-based molecules (for example compounds **1**[7] and **2**[8], respectively), terpyridines (compound **3**[9]), bile acid derivatives (compound **4**[10]), and crown ethers (compound **5**[11]), to name a few. This is one aspect, which makes the field of supramolecular gels fascinating. In addition, the self-assembly process itself is somewhat mysterious and there is no final answer to why and how the gels are formed in detail. The weak molecular forces responsible for the gel formation are different depending on the compound(s) and solvent(s) used. Furthermore, the design of gelator molecules has proven to be challenging, because it is almost impossible to predict how the designed molecule will behave in nature. As a testimony to this, many gelators have been discovered serendipitously. However, as knowledge has accumulated and new techniques are available, it is possible to derive new, designed gelators from serendipitously discovered ones by different methods.[12] As a result, myriad of supramolecular gel systems have applications today, for example in regenerative medicine[13,14] and environmental remediation,[15–18] owing to the diversity of the gel forming compounds and their properties such as mouldability, self-healing, and 3D-printability.[19]

As materials, gels are well-known in everyday life from divergent applications such as soft contact lenses, gelatine, and silica gel. Gels are remarkable viscoelastic solid-like materials: they consist of mainly solvent.[1] Typically 99 % of the supramolecular gel is solvent, which means that only 1 % of the gel consists of the gelator. Supramolecular gels are commonly formed by low molecular weight gelators (LMWGs) giving rise to a network of fibres immobilising the solvent used. The immobilization of the solvent or solvent mixture occurs *via* capillary forces and surface tension.[4]

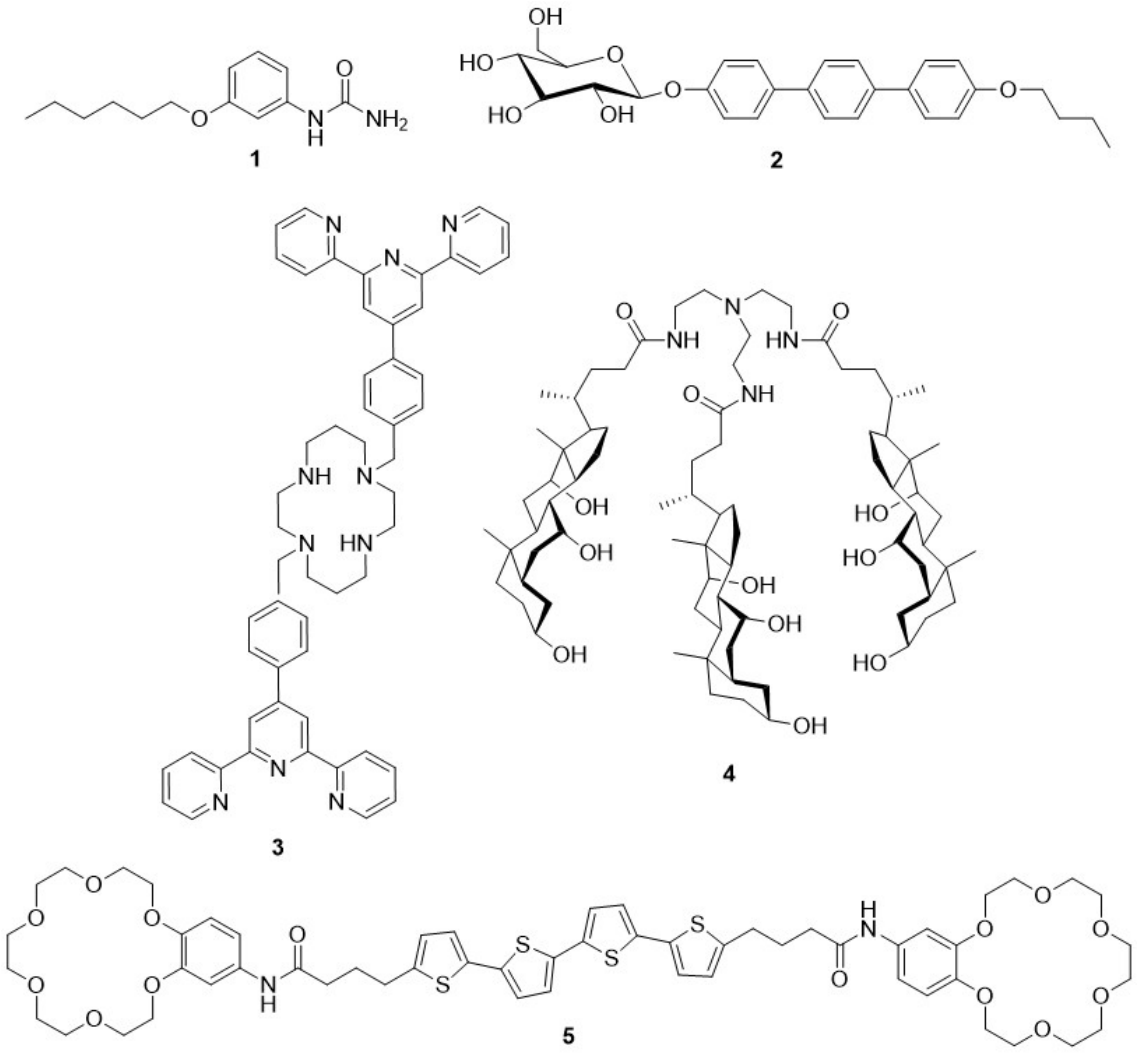


FIGURE 1 Different molecules discovered to act as gelators: urea-based compound **1**, sugar-based compound **2**, terpyridine-based compound **3**, bile acid based compound **4**, and crown-ether containing compound **5**.

Weak molecular interactions governing the supramolecular gel formation are for example van der Waals interactions, metal coordination, π-π-interactions, and hydrogen bonds.[1–4,6] Additionally, gelation based on halogen bonding has been discovered.[20] Gels formed due to these weak interactions are generally thermoreversible and, on some occasions, thixotropic.[1–4,6,19] The formation of the fibrous network entrapping the solvent molecules is perceived to occur hierarchically, leading to the formation of long polymer-like fibres from gelator molecules. These long fibres entangle together and trap the solvent molecules. In time most supramolecular gels disassemble by the precipitation or crystallization of the gelator. A schematic representation of the gelation process is illustrated in the Figure 2.

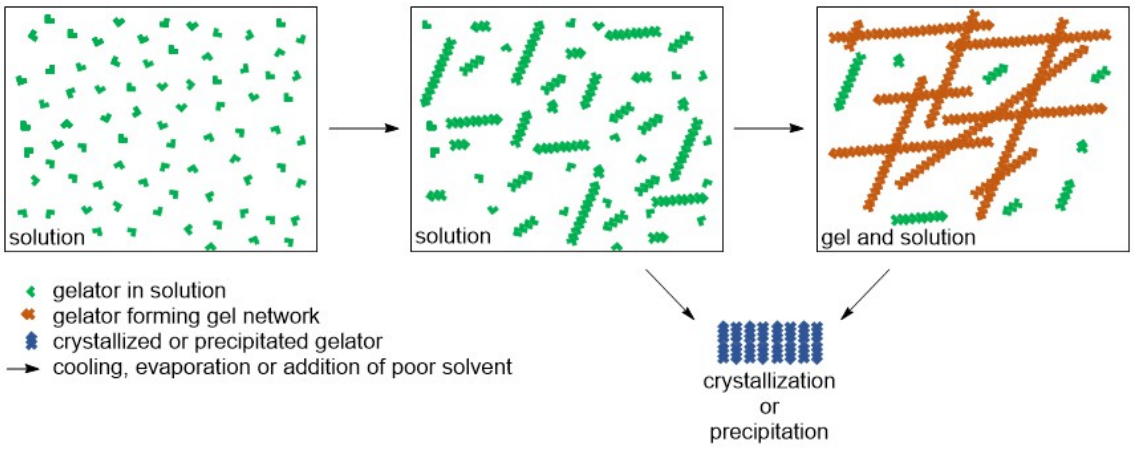


FIGURE 2 Simplified depiction of the process of gel network formation.

Supramolecular gels can be categorized in different classes, most generally by the solvent in which the gel is formed.[6] The most common types of gels are organogels and hydrogels. Organogels contain organic solvent and hydrogels are formed in water or a mixture of water and other solvent. Xerogel refers to a dried gel, which is commonly used in the study of the gel network structure. Ionogel is somewhat rare type of gel that form in ionic liquids and possess intriguing electrochemical properties.[2] Additionally, aerogels can be seen as a one class of gels.[21] In aerogels, the solvent is replaced by a gas leading to materials with divergent densities and, on some occasions, ultralight materials.

1.1.1 How supramolecular gels are studied?

Methods of studying supramolecular gels have evolved tremendously over last three decades. Wet and dried gels can be studied with multiple methods, all providing divergent information of the gel and the network responsible of the formation of the gel.

The gel network itself can be examined with AFM, SEM, TEM, as well as cryo-SEM and cryo-TEM.[4,22] Depending on the method chosen, either dried or wet gel is used. All the imaging methods provide a detailed picture of the gel network. Especially cryo-SEM and cryo-TEM are useful methods for observing the gel network because the wet gel can be studied.[1,4] By this method, the actual gel network can be observed as it has not been affected by the slow drying process of the sample. AFM provides more three-dimensional depiction of the gel, but this technique is not as widely used as SEM. The limitation of previously mentioned imaging methods is that only a very small area of the gel network is seen simultaneously. In addition, when utilizing AFM, the tip of the apparatus can cause defects into the soft gel material which might cause flattening of the gel network structures.[22] Other methods of detecting the gel network are confocal microscopy and small-angle X-ray or neutron scattering which provide infor mation of the whole bulk sample *in situ*.[4,22] Additionally, traditional light microscopes can be used to examine the gel network. Figure 3 illustrates the divergent information achieved by utilizing AFM, SEM and TEM techniques.[23]

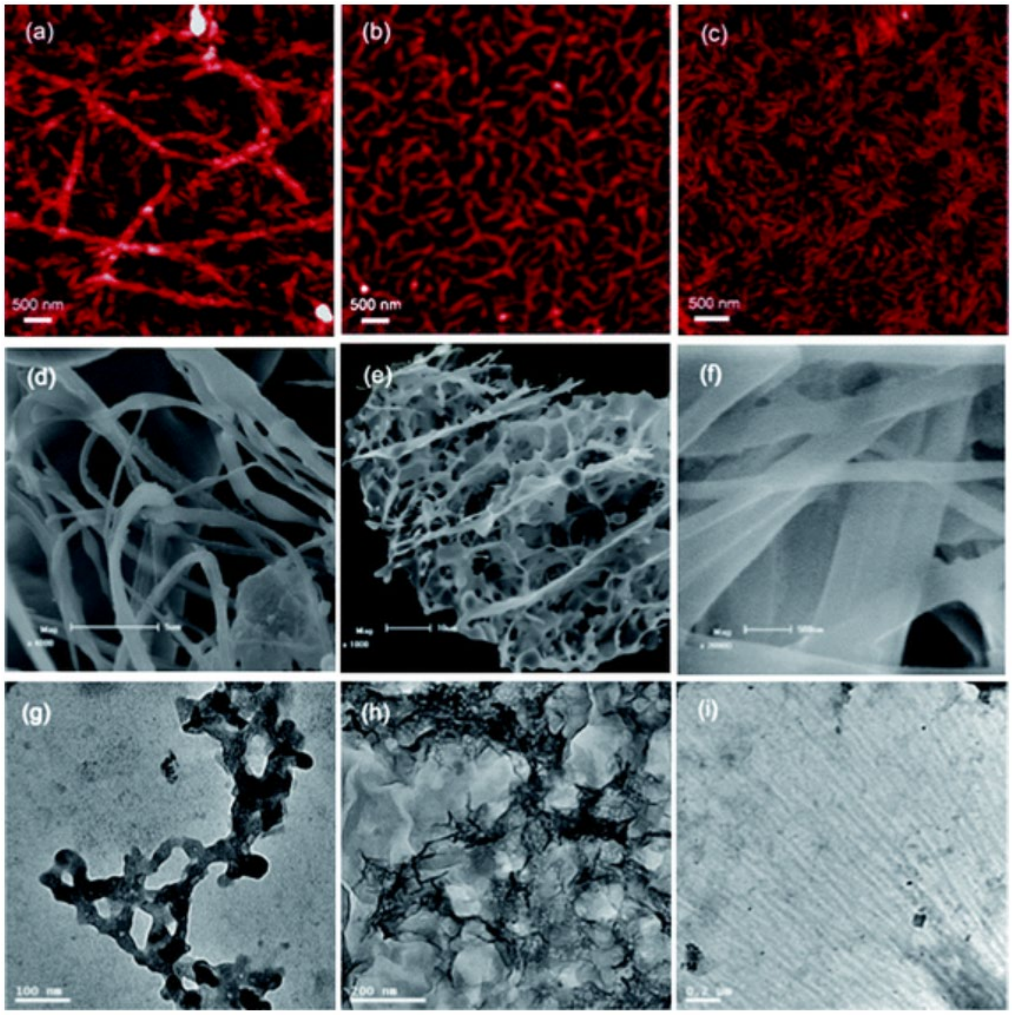


FIGURE 3 First row: AFM images of wet gels. Second row: SEM images of xerogels. Third row: TEM images of xerogels. Republished with permission of Royal Society of Chemistry, from Supramolecular binary hydrogels from calixarenes and amino acids and their entrapment–release of model dye molecules, J. Zhang et al. Soft Matter, 7, 2011; permission conveyed through Copyright Clearance Center, Inc.

By utilizing rheology, the mechanical properties of the gel are revealed.[4,22] When the gel is under different physical stress in the rheology measurement, information of the gels stability and reversibility can be achieved.

Solid-state NMR allows the study of the wet gel as well as the dry gel as powder and solid gelator compound by itself.[22] This method provides information for example of the hydrogen bonding in wet and dried gel state. In addition, X-ray diffraction is used to study the solid-state interactions. With powder diffraction it is possible to visualize the molecular interactions in a xerogel[24] and the crystalline gelator can be explored with single crystal X-ray diffraction.[22] However, there has been a debate whether there is a link between crystallization and gelation process or not.[4]

Molecular interactions are also studied by using IR spectroscopy, UV-Vis absorption, and fluorescence.[4,22] In addition, circular dichroism can be utilized.

All of the techniques have their limitations due to the technical features, for example it is difficult to measure fluorescence from a turbid sample.

There are many other methods by which supramolecular gels can be studied with, but they are quite rare.[22] For example at least two divergent methods can be utilized for determining the gel-sol transition temperature, which both have limitations that lead to inaccuracy of the determination. And naturally a myriad of instruments and methods for the study of the gelator molecules in liquid and solid states.

1.1.2 Applications of supramolecular gels

Applications of supramolecular gels cover multiple areas of modern life. As various types of molecules are capable to self-assemble into gels and a myriad of supramolecular gels are stimuli responsive, innumerable number of divergent applications have emerged. Supramolecular chemistry provides an extraordinarily broad canvas for the design of new materials and technological applications based on self-assembly.

Regenerative medicine is a fascinating area concerning the applications of supramolecular gels. Generally the applications are based on hydrogel systems due to the biocompatibility of water. Supramolecular gels in regenerative medicine are considered as smart materials.[13] The gel systems in this field are able to undergo *sol-gel* and/or *gel-sol* transitions as a result of minuscule changes in the environment. The stimuli which cause beforementioned changes in the gel are categorized into three classes: chemical, physical, and biological stimuli. Chemical stimuli include changes in the pH, acid-base reactions, and metal ligations to name a few. Light, temperature, and electric and magnetic fields (the two latter being rarer) belong to the physical stimuli. Biological stimuli are considered to be mainly effects produced by enzymes. Additionally, there are supramolecular gel systems capable of responding to multiple stimuli.

Numerous promising applications are based on hydrogelation of amino acids and peptides in the field of tissue integration.[13,14,25] These divergent hydrogels have been observed to function in regeneration of nerves[25] and cartilage repair,[25,26] for example. Various applications of this type of supramolecular gels arise from many advantages that lie on the background.[14] When looking into the aspect of tissue rejection, the protein-based hydrogels have been observed to be immunobiologically inert. Since the compounds forming the hydrogels are usually biocompatible, the resulting gel is likely to be biocompatible as well.[27] When the peptide-based gel is degraded, it is excreted in urine as amino acids and as protein metabolism products.[14] And, most important of all, the gel system provides a perfect (pseudo *in vivo*) environment for cell growth, differentiation, and migration. This is due to the gel network which is similar in size when compared to the extracellular matrix. Furthermore, other types of compounds are capable to form gel systems having possible applications in regenerative medicine, such as hyaluronic acid.[28]

Hyaluronic acid is a high molecular mass polysaccharide, which is found in particular in the extracellular matrix of soft connecting tissues and is used in

multiple medical applications.[29,30] To most people hyaluronic acid is mostly known for its use in cosmetic surgery procedures[31] but it is exploited in other medical applications as well. Drug delivery, especially controlled drug delivery, is intriguing medical application of supramolecular gels. The release of the drugs is based on the degradation of the gel system by enzymatic response or pH.[14,27] Injectable systems have been known for decades, one quite recent example is a hyaluronic acid-tyramine hydrogel which was observed to successfully release therapeutic proteins.[32] Injectable hydrogels are also studied regarding antitumor therapy.[33] A polypeptide hydrogel containing doxorubicin, a very effective antitumor drug, was found to form at 37 ˚C (normal body temperature). The same system was a solution at 25 ˚C which makes the system easy to use. In test performed with mice, the results were promising as in some cases the tumours in mice were completely removed by this method. Controlled drug release can be achieved also by mixing of two supramolecular hydrogelators.[34] Naproxen (Figure 4, compound **6**), a non-steroidal anti-inflammatory agent, was observed to be released from the two-component hydrogel of DBS-$CONHNH_2$ and MBS-CO_2Me (Figure 4, compounds **7** and **8**, respectively) depending on the pH. In lower pH, naproxen was released, making the system studied attractive possible application for orally administered drug that is released in the small intestine to minimize the side-effects.

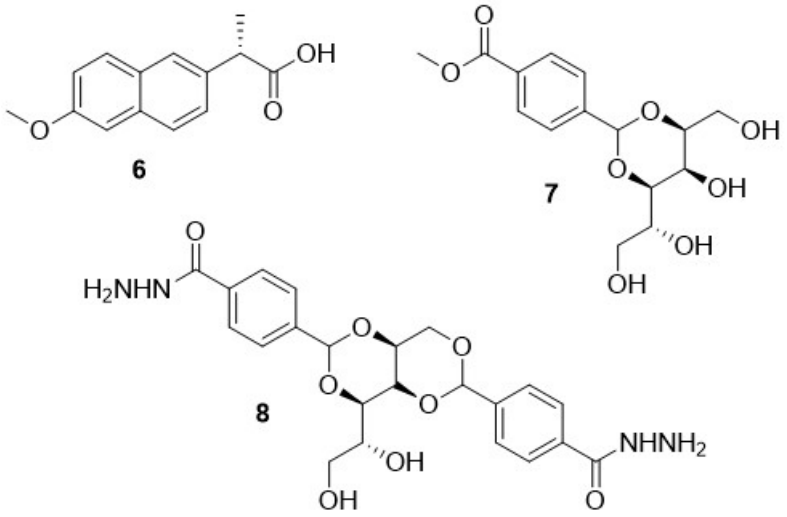


FIGURE 4 Compounds responsible of forming the two-component gel capable of releasing Naproxen.

Divergent gels have been used as media for single crystal growth for over a century.[3] The supramolecular gel can aid the crystal growth by two different methods. The gel can act as an inert matrix for crystallization by, for example, limiting the nucleation. Additionally, the gel can take more active part in the crystallization process by impacting on the polymorphism. Foster *et al.*[35] were able to obtain a metastable polymorph of ROY, a model drug molecule (Figure 5), from a supramolecular gel formed by bis(urea)-based compound **10** (Figure 5). The structure of the gelator was designed in a manner which produced gel

fibres chemically mimicking ROY. This technique can provide significant improvements to obtaining pure enantiomers of drug molecules which are extremely difficult to crystallize in the desired conformation. Furthermore, multicomponent supramolecular gels can be utilized in the crystallization of drug molecules.[36] Supramolecular gels have been utilized also in the growing of single-crystal halide salt nanowires upon drying of the gel system.[37]

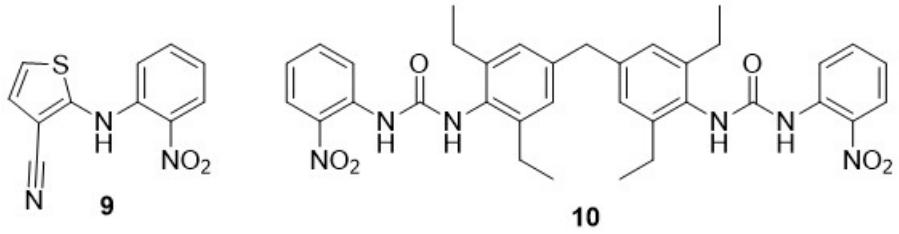


FIGURE 5 Model drug molecule ROY (compound **9**) and bis(urea)-based gelator (compound **10**).

Gels formed with metal ions are a one subgroup of supramolecular gels that has gained interest increasingly due to the possible hi-tech electrical applications.[1,14,19,38,39] By utilizing metal ions, the resulting gel can also possess intriguing magnetic or photophysical properties. On the other hand, the metal ion can be relatively inert part of the formed gel's structure, enabling the formation of the gel or making the gel structure more stable. One interesting example of how the metal cations enforce the formed supramolecular gel when compared to the gel formed without metal ions is a gel which is composed of two compounds in DMSO.[40] The gel self-assembled as a result of oligomerization of calix[4]arene hydrazine derivative and 18-crown-6-ether derivative functionalized with terminal aldehyde (Figure 6 compounds **11** and **12**, respectively). When Cs^+ was present in the gel system, the gel was observed to be significantly stronger than the gel without Cs^+ due to the sandwich complex formed with the 18-crown-6-ether derivative. The gels with and without Cs^+ were also able to hold their shape when moulded into divergent shapes, as can be seen from Figure 7. Additionally, Cs^+ containing gel was found to be electrically conductive, the conductivity of the gel being proportional to the amount of the Cs^+ in the gel.

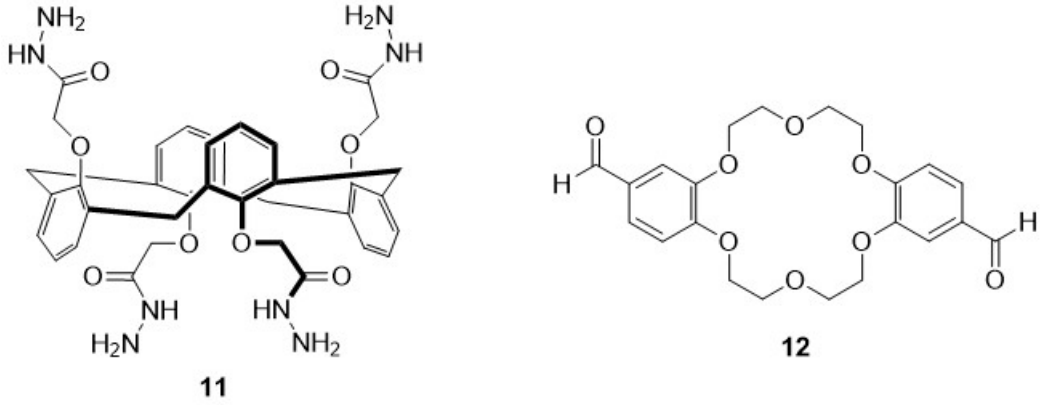


FIGURE 6 Compounds **11** and **12** capable of forming supramolecular metallogels.

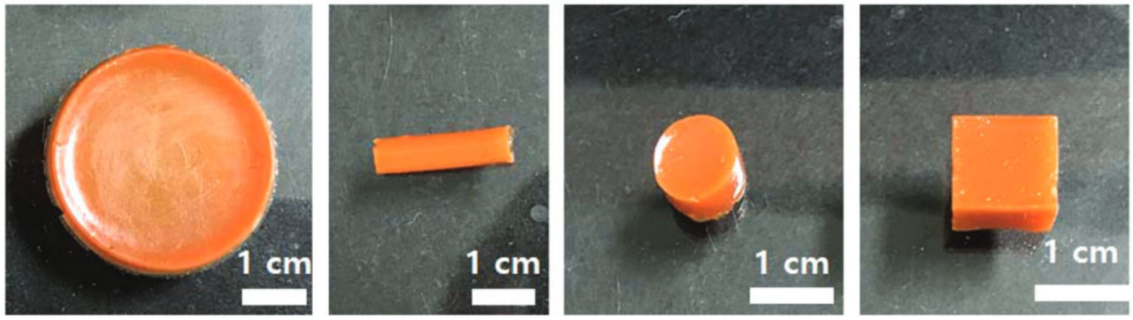


FIGURE 7 Supramolecular gels formed by compounds **11** and **12** in divergent vessels holding their shape. Republished with permission of Royal Society of Chemistry, from A crown-ether-based moldable supramolecular gel with unusual mechanical properties and controllable electrical conductivity prepared by cation-mediated cross-linking, J. Park *et al.* Polym. Chem. 9, 2018; permission conveyed through Copyright Clearance Center, Inc.

Environmental point of view has been increasingly the hot potato in the field of supramolecular gels. The removal of divergent pollutants from water is essential in modern industrialized world.[18,41] Supramolecular gels can provide better methods for removal of dyes, divergent oils, heavy metals, anions, and even chemical warfare agents from water.[16,18] The advantage in utilizing gels instead of more conventional methods, such as zeolites or sawdust, are profound. The use of supramolecular gels reduces the amount of dangerous waste because the gel systems are generally regenerated after use and not stored as a huge amount of sludge. The regeneration of gels leads also to recovery of pollutants which can be then used again in industrial processes. Selectivity towards the pollutant is another important aspect, in addition to the capacity to bind the pollutant in question. Furthermore, several kinds of pollutants could be removed from wastewater simultaneously by using different hydrogelators. When regarding commercial applications, the low costs of synthesis and regeneration are essential. One interesting potential low-cost application for recovery of oil spills was discovered by Bear *et al.*: a new group of urea-based organogelators, two of which

were able to efficiently gelate dirty engine oil in the studies.[17] Regarding dye absorption from wastewater, a promising ionogel system for dye absorption from wastewater was developed.[41] Didodecylimidazolium salt-based system (Figure 8, compound **13**) was able to remove 95 % of the Rhodamine B dye (Figure 8, compound **14**) in just 10 minutes when the ionogel was loaded into a column. Furthermore, the system can be utilized in 20 cycles and after partial regeneration additional 15 cycles.

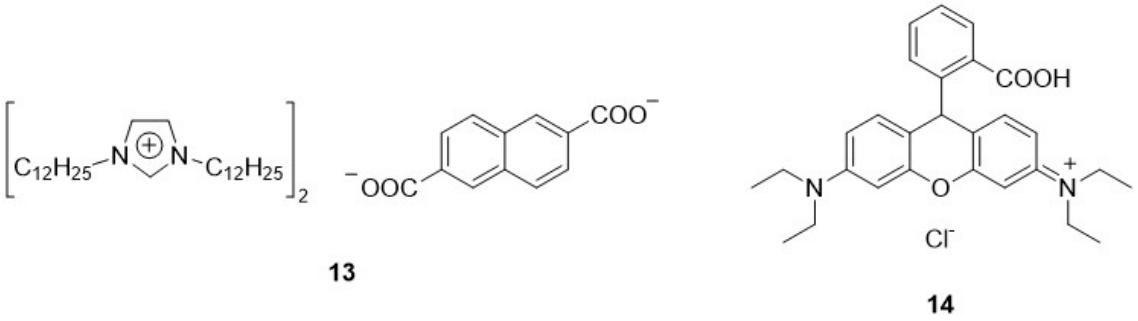


FIGURE 8 Didodecylimidazolium-based salt (compound **13**) and Rhodamine B (compound **14**).

1.2 Bile acids

Bile acids are intriguing research objects with respect to multiple point of views. They are intrinsic to human bodies because they are formed in liver through complex biosynthesis, thus bile acids are biologically important steroidal molecules.[42–50] For a long period of time bile acids were thought only to assist in emulsion and absorption of fats and liposoluble vitamins. But, since the 1950's the understanding of the complexity of the biological roles of bile acid and their salts have increased tremendously. Bile acids have been discovered to play important roles in divergent biological functions in human bodies and in several diseases as well.

Steroids are the main group of compounds in which bile acids as acidic steroidal compounds belong to.[42,43] Bile acids are amphiphilic due to their structure: hydrophilic α-side containing hydroxyl groups and hydrophobic β-side containing methyl groups (Figure 9). It is from this structural characteristic that arises multiple fascinating biological, physical, and chemical properties of bile acids, such as the capability to form mixed micelles with cholesterol. Bile acids are also functioning as inflammatory agents and signaling molecules.[51] Additionally, even though the structural differences between divergent bile acids are seeming ly small, the effects on the properties are major.[43] Figure 10 presents the common bile acids in humans and their structural differences.

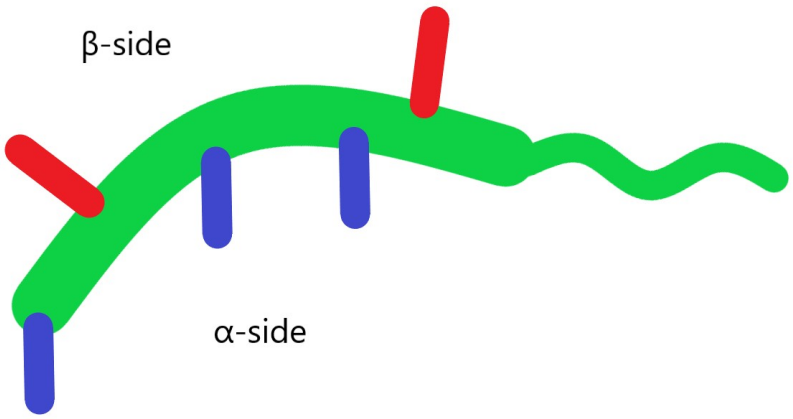
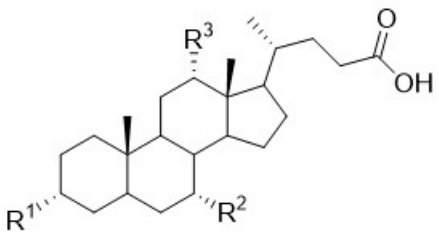


FIGURE 9 Schematic representation of the amphiphilic nature of bile acids. Colour code: steroidal backbone and side chain (green), methyl groups (red), and hydroxyl groups (blue).



15 R^1 = H, R^2 = H, R^3 = H
16 R^1 = OH, R^2 = H, R^3 = H
17 R^1 = OH, R^2 = H, R^3 = OH
18 R^1 = OH, R^2 = OH, R^3 = H
19 R^1 = OH, R^2 = β-OH, R^3 = H
20 R^1 = OH, R^2 = OH, R^3 = OH

FIGURE 10 Most abundant bile acids in humans: cholanic acid (**15**), lithocholic acid (**16**), deoxycholic acid (**17**), chenodeoxycholic acid (**18**), ursodeoxycholic acid (**19**), and cholic acid (**20**).

The biosynthesis of divergent bile acids can occur *via* two main paths in mammals but, to date, there is still uncertainty of the exact mechanism of bile acid formation and the regulation of the biosyntehsis.[42–44,52] After the biosynthesis in the liver the bile acids can be modified also by gut bacteria, for example. In humans, nearly all of the bile acids are conjugated to two amino acids (25 % to taurine and 75 % to glycine) which makes the bile acids more water soluble. The regulation of the biosynthesis is complex as multiple factors have an impact on it, such as the amount of bile acids returning to the liver after enterohepatic circulation.[51]

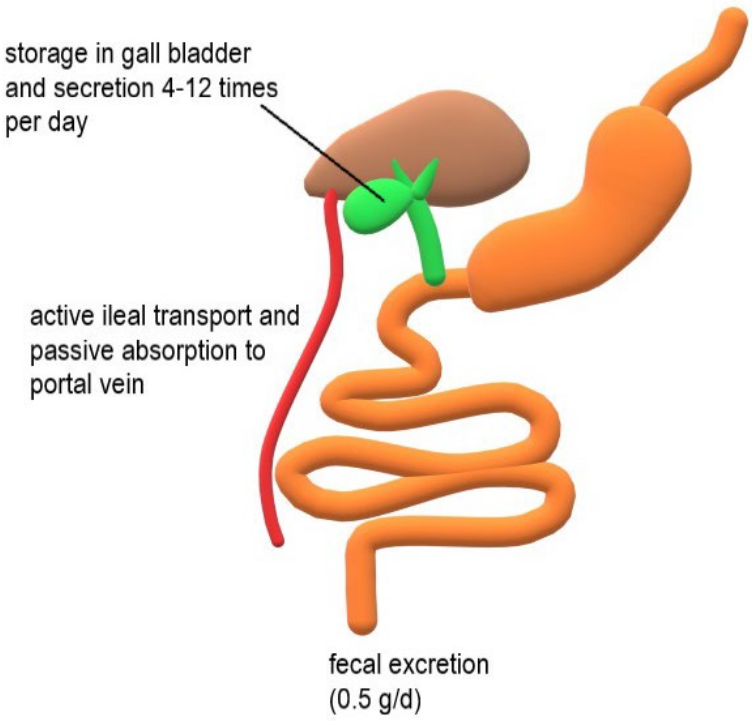


FIGURE 11 Simplified representation of enterohepatic circulation.

Enterohepatic circulation (Figure 11) is the route by which the bile acids circulate in human bodies.[42–44,46,51] First, cholesterol is converted into bile acids in the liver. Then bile acids are transported with carrier proteins to gall bladder, from which they are excreted to the small intestinal lumen when food is consumed. Bile acids assist in the digestion of fats and fat-soluble vitamins and in lowering the cholesterol level. Additionally, antimicrobial effects by bile acids have been observed in the small intestine. Even transport of heavy metal ions in bile acid micelles has been discover. Up to 95 % of the bile acids circulate again and are stored back to the gall bladder as the biosynthesis consumes a substantial amount of energy. In total a pool of 3 g of bile acids is recycled, from which 02.–0.6 g is synthetized daily. This reuse of bile acids is one aspect by which the enterohepatic circulation is regulated. The biosynthesis of bile acids and oral uptake of bile acids also effect on the enterohepatic circulation, in addition to other factors.

Naturally, bile acids and compounds derived from them have applications in medicine.[53–55] These applications range from drugs,[52,56,57] nutrients,[52] and versatile biomaterials[49,58–60] to carriers for drugs[61–63] and antimicrobial agents.[64,65] Many of the applications take advantage of the endogenous transport systems of bile acids in addition to the unique chemical, physical, and biological features of them. Since bile acids are found in multiple locations of the human body, they and their derivatives are promising in targeting drugs and for developing new drug releasing systems for a myriad of medical conditions ranging from liver diseases[66–69] and diabetes[70–73] to cancer.[74–76]

In supramolecular chemistry bile acids and their derivatives have been studied extensively. Because of the rigidity of the steroidal backbone, chirality, amphiphilicity, and relatively facile side chain modification bile acids are attractive starting materials regarding supramolecular chemistry. Therefore, it is no surprise that bile acids and their derivatives are widely utilized in a large quantity of divergent supramolecular applications.[2,53,62,77]

One intriguing field of supramolecular applications based on bile acid systems is the formation of different nanostructures. Quite recently cationic and anionic species of deoxycholic acid (Figure 12, compounds **21** and **22**, respectively) which formed divergent assemblies were discovered.[78] Compound **21** formed tubules as helically folded ribbons and compound **22** formed mostly partially folded rectangular lamellae. When the two compounds were mixed and the anionic compound was present at larger concentration, the formed structures were mostly tubular. Another fascinating example of nanostructures are supramolecular chemical springs formed by lithocholic acid (Figure 13 a) in water.[79] In 0.1 M NaOH solution lithocholic acid self-assembled into vesicles which then gradually fused together into hollow tubes until there was not any vesicles left (Figure 13, b-d). As the tubes grew in length, the shape transitioned from straight to left-handed spirals. In pH 12 the spirals were stable, but when the pH was adjusted to 7.4 with HCl uncoiling of the spirals was detected. The coiling of the tubes was speculated to be a result of the ionization of the carboxyl groups as the pH is set to 12. This disintegrates the hydrogen bonds between lithocholic acid molecules making the tubes less elastic leading to the formation of spiral tubes.

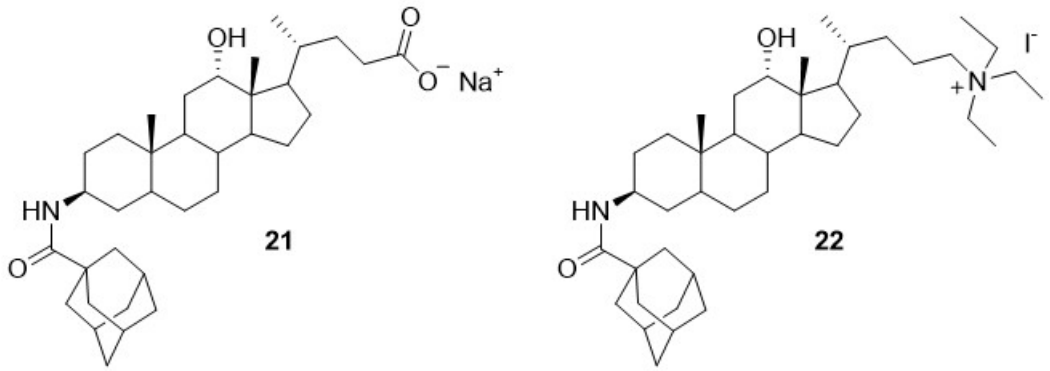


FIGURE 12 Anionic (compound **21**) and cationic (compound **22**) derivative of deoxycholic acid.

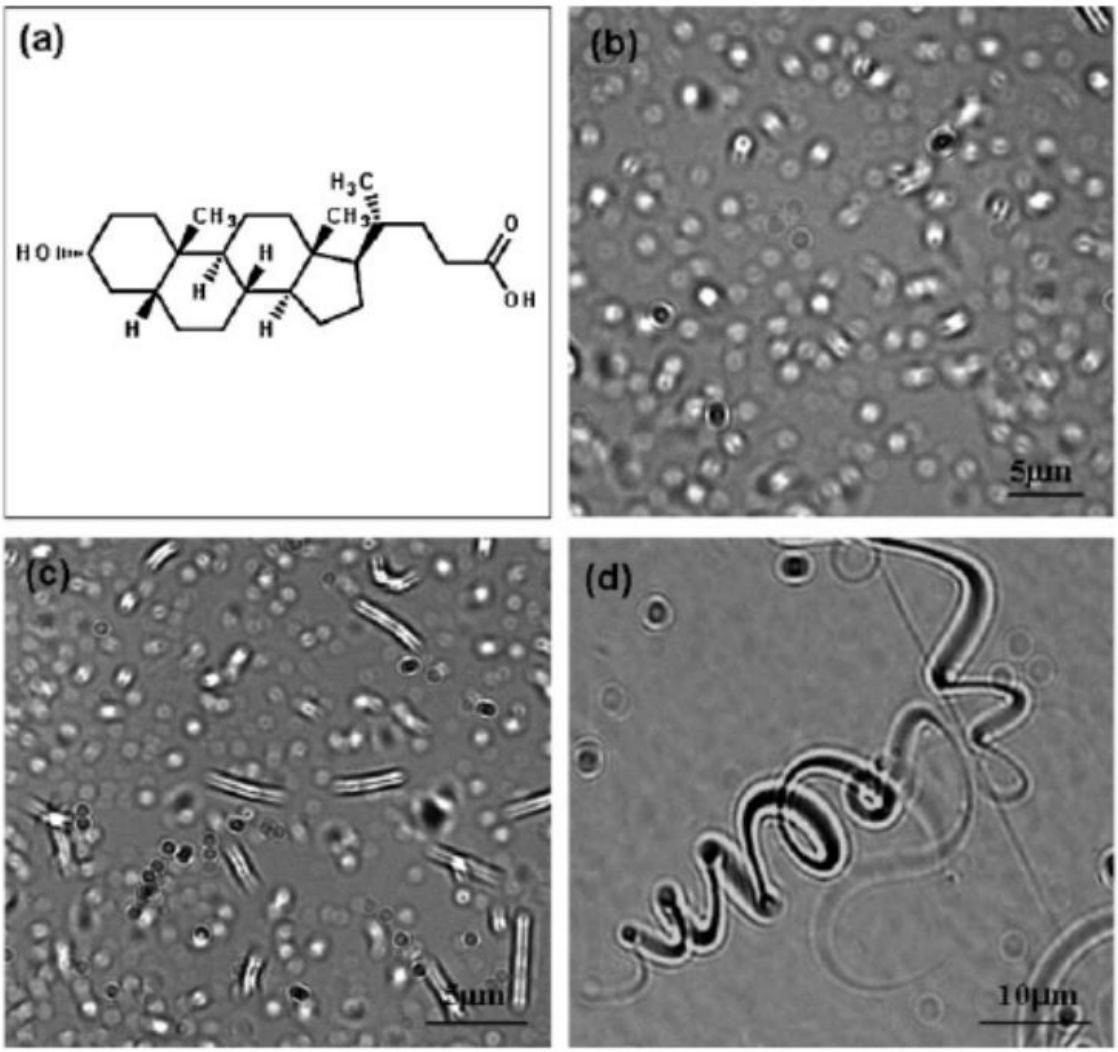


FIGURE 13 Lithocholic acid (a) and the optical microscopy images of self-assembled lithocholic acid in water solution (pH 12) at room temperature after 2 h (b), 6 h (c), and 3 days (d). Reproduced with permission from ref. 79. Copyright (2010) Wiley.

1.3 Supramolecular gels formed by bile acid derivatives

Bile acid derivatives cover a wide collection of divergent compounds with one common feature: the steroidal backbone consisting of three six membered rings and one five membered ring connected to each other in a unique fashion producing a curved structure. The first example of bile acid based supramolecular gel, a sodium cholate (Figure 14) gel, was discovered in 1914 by Schryver.[80–82] After that, bile acid based gels were virtually forgotten for decades until their research emerged again in larger volume in the 1980's. Since then, a myriad of gel systems containing divergent types of bile acid derivatives have been found and utilized in various applications.

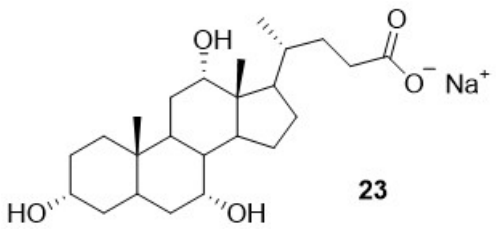


FIGURE 14 Sodium cholate salt.

Both charged and neutral bile acid based compounds have been observed to act as hydrogelators over the several decades of research. For example, cationic and neutral deoxycholyl and cholyl derivatives depicted in Figure 15 showed differing self-assembly properties in aqueous solutions.[83] Compounds **24** and **25** formed hydrogels but compound **26** was not able act as a gelator. Both gel-forming cationic compounds **24** and **25** were able to form gels in water in the presence of NaCl with different concentrations of the salt and the compound in question. Compound **25** formed gels in pure water. Instead compound **24** required NaCl or other salt, such as NaBr or LiCl, to gelate water. Another interesting dissimilarity between compounds **24** and **25** was observed when investigating the thermal stability of the formed hydrogels. The gel of compound **25** in 1 M NaCl solution showed identical thermal stability regardless of the concentration of the compound **25**. Under same conditions, the hydrogels of compound **24** experienced enhanced thermal stability with increasing concentration of compound **24**. Similar effect was detected for 1 % (w/v) gels of compound **24** with increasing the amount of NaCl in the gel system. On the contrary, compounds **27–29** containing neutral hydrophilic side chains were insoluble to water and only compound **27** was able to form hydrogels when a polar solvent (MeOH, DMSO, *etc.*) was added. This was speculated to arise from the importance of the amide bond for the gelation process and the number of hydroxyl groups on the steroidal backbone. In a following study compound **27** was compared with corresponding compound **30** with two hydroxyl groups in the side chain.[84] Compound **30** was not as good a gelator as compound **27**: weaker gels in fewer aqueous solutions were observed by compound **30**.

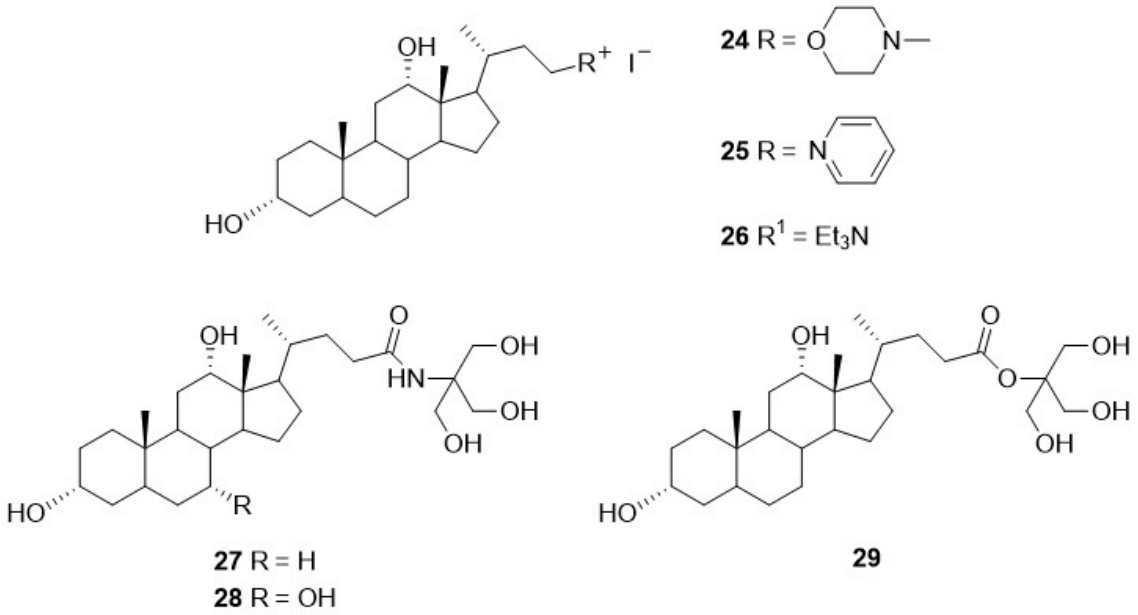


FIGURE 15 Neutral and cationic bile acid derivatives.

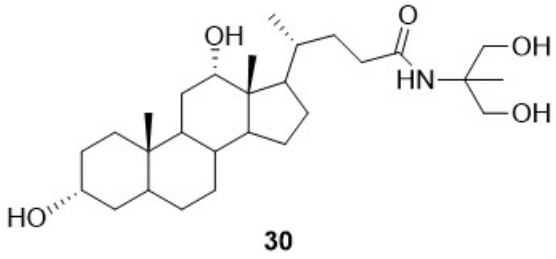


FIGURE 16 Deoxycholyl derivative with neutral hydrophilic side chain.

Dimeric bile acid derivatives have also been observed to self-assemble into gels, for instance compound **31** (Figure 17) containing dehydrocholic acid moieties.[85] The compound **31** itself had poor solubility in water but it was able to dissolve in alcohols, for example methanol and glycerol, and in water when mixed with potassium hydroxide. Gelation occurred only in water by this method, after the conversion of compound **31** into a salt. The hydrogel was observed to be thermoreversible and very stable, no phase separation was detected within six months. FT-IR measurements of the xerogel revealed dipole-dipole and electrostatic interactions in addition to several hydrogen bonds were responsible for the hydrogel formation. This led to the conclusion that no gelation in alcohols occurred due to the weakening of the interactions leading to gelation by solvent molecules. In Figure 18 the formation process of gel network is presented. The compound **31** forms one-dimensional fibres which curve and intertwine forming three-dimensional gel network.

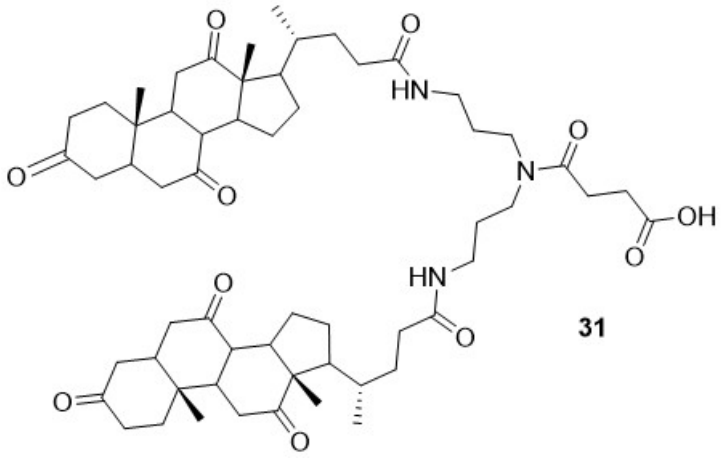


FIGURE 17 Dimeric bile acid derivative containing dehydrocholic acid.

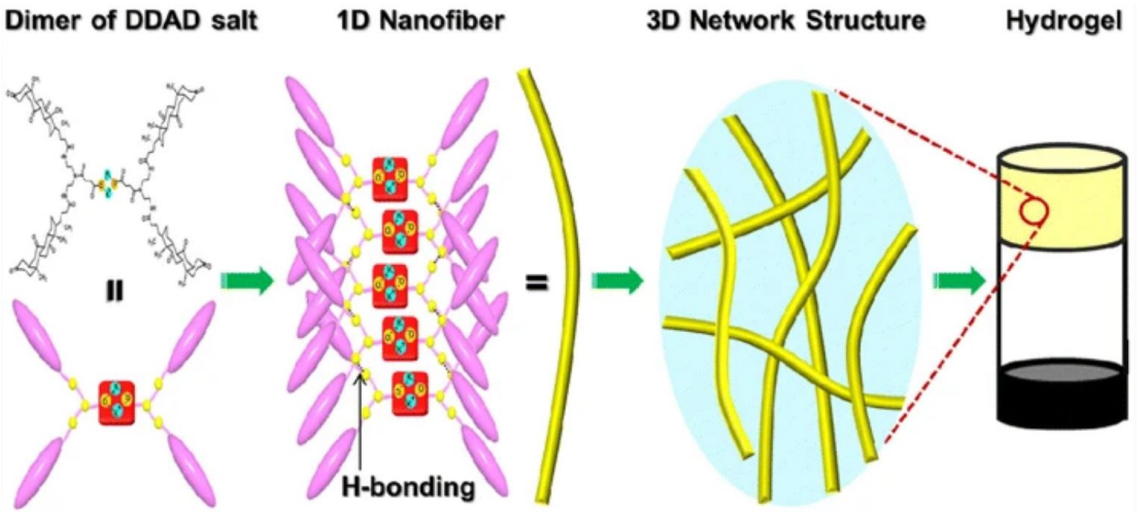


FIGURE 18 Schematic representation of the gel formation by compound **31** in basic water solution. Reprinted by permission from Springer. Colloid Polym. Sci. A novel hydrogelator based on dimeric-dehydrocholic acid derivative, H. Yang, P. Qi, and H. Zhao, Copyright (2018).

Among the steroidal compounds capable of forming metallogels examples of bile acid based gelators are rare.[86] One intriguing example of a such metallogelator is compound **32** (Figure 19) which formed metallogels with copper salts.[87] The deoxycholyl derivative **32** containing a pyridyl moiety was observed to form gels in aqueous solvent mixtures with 30–50 % of organic solvent in the presence of a copper salt, whereas compound **32** by itself did not promote gelation. The copper salt used had major effects on the gelation and the stability of the gels. With $CuCl_2 \cdot 2H_2O$ no gelation was observed. When utilizing $CuSO_4 \cdot 5H_2O$ gels were formed more often than with $Cu(NO_3)_2 \cdot 3H_2O$. On the contrary, gels with $Cu(NO_3)_2 \cdot 3H_2O$ were more stable. The metallogels were also responsive to multiple stimuli. Chemical stimuli resulted in the degradation of the gel. The gels

started to disassemble immediately after the addition of solid disodium salt of EDTA, pyridine, triethylamine, or aqueous ammonia to the surface of the gel (Figure 20). In addition, the metallogels of compound **32** were observed to possess reversible redox-responsiveness (Figure 21). Ascorbic acid was used to reduce the Cu^{2+}-ions to Cu^{+}-ions which resulted in yellow solution after heating. After cooling and shaking the solution was green and included green precipitate. The addition of nitric acid and heating produced clear light blue solution which, after sonication, formed a gel.

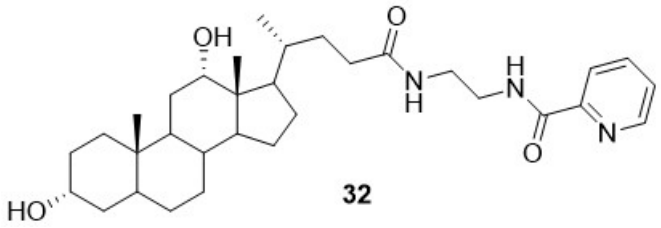


FIGURE 19 Conjugate of deoxycholic acid and picolinic acid (compound **32**).

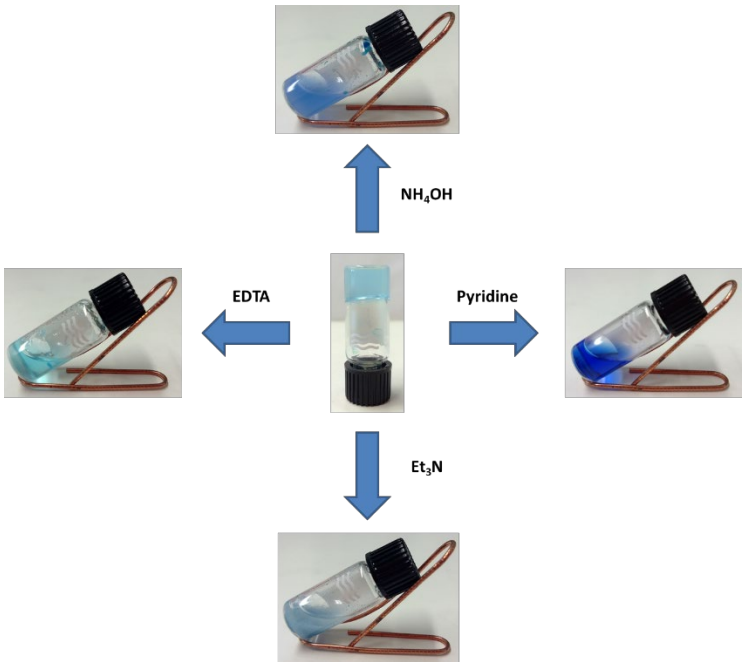


FIGURE 20 Copper metallogels of compound **32** showing responsiveness to chemical stimuli. Reprinted from ref. 87.

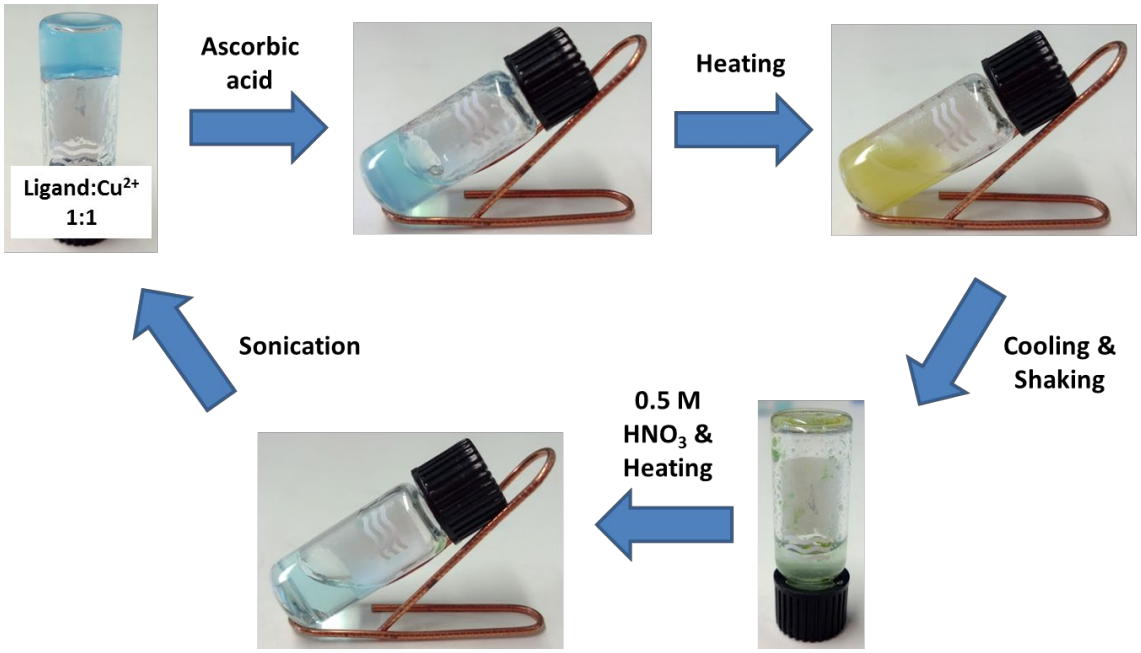


FIGURE 21 Redox-responsiveness of the metallogels by compound **32**. Reprinted from ref. 87.

1.4 The effect of structure on the gel formation and the properties of supramolecular organogels formed by bile acid amides

Several bile acid amides have been discovered to act as gelators in organic solvents with and without of the presence of added compounds, such as charge transfer agents[88] and gold nanoparticles.[89] In addition, formation of hydrogels is commonly observed with bile acid derivatives.[77,85,90,91] Herein the focus is on bile acid amide derivatives capable to act as organogelators themselves.

The earliest example of bile acid based organogelator was discovered by Hishikawa *et al*. in 1998.[92] Cholyl derivative with *iso*-propyl side chain (Figure 22) self-assembled into transparent gels in various solvent mixtures. The concentration of compound **33** was high: 70 mg in 0.6–1.2 mL of solvent mixture (0.2 mL methanol and 0.4–1.0 mL other solvent). The added solvent inducing gel formation was aromatic, such as benzene and mesitylene. Interestingly, one gel formed in rapeseed oil. In other solvent systems different fashion of self-assembly was observed. Inclusion crystals formed for example in ethanol, whereas single crystals without solvent molecules in the crystal lattice were observed in THF in addition to other solvents.

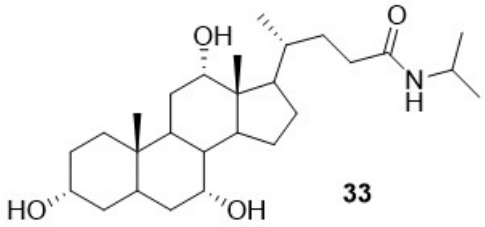


FIGURE 22 Cholyl derivate **33**.

Löfman *et al.* studied a variety of amino- and hydroxyalkyl amides (Figure 23) with respect of gelation.[93] Each compounds gelation ability was tested in 36 divergent solvents, and 22 gel systems were obtained. Self-assembly occurred mainly in aromatic solvents, such as anisole and *m*-xylene, by lithocholyl derivatives **34**, **37**, and **40**. Compound **41** with deoxycholic acid backbone formed one partial gel in anisole. The hydroxyalkyl amides **43** and **44** were not as readily soluble as the aminoalkyl amides **34–42**, hence the compounds were not able to form gels. Only one partial gel in anisole by compound **43** was formed. Evidently, the two hydroxyl groups in the side chain prevent gelation in organic solvents. In addition, all but one gel were formed by lithocholyl derivatives containing one hydroxyl group in the steroidal part of the molecule.

In the case of lithocholyl derivatives **34**, **37**, and **40**, a correlation with the gel formation ability and the side chain length was found.[93] Longer side chain resulted in self-assembly to gel systems in larger number of solvents in the order of **40>37>34**. Under similar conditions compounds **34**, **37**, and **40**, were observed to form two, six, and ten gel systems, respectively. Additionally, compound **34** formed a partial gel in DCM, but the gel composition was speculated to be over 1 % (w/v) due to the low boiling point of the solvent.

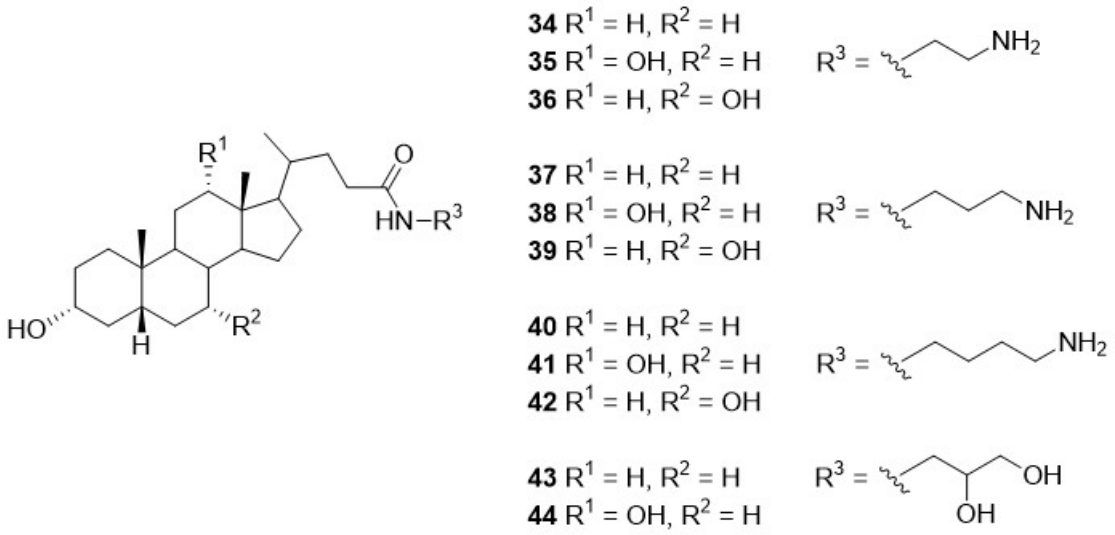
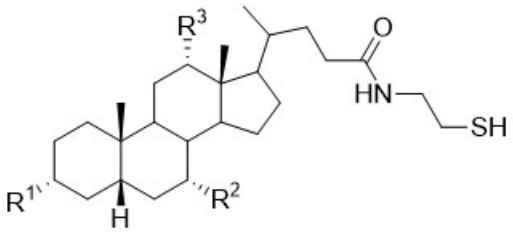


FIGURE 23 Amino- and hydroxyalkyl amides.

Structurally similar steroidal derivatives of compounds **34–36**[93] containing cysteamine side chain (Figure 24) were studied by Noponen *et al*.[94] Apart from a weak gel in benzene by compound **45** (containing lithocholyl backbone), no gelation induced by compounds **45–48** was observed in the series of 37 different solvents, including aqueous mixtures, studied. This was due to the insolubility, precipitation or too efficient solubility of the compounds in selected solvents. Hence, two hydroxyl groups in the steroidal backbone hindered gel formation. Interestingly, precipitation from almost all aromatic solvents was observed for compounds **45–48**.

Compounds **49** and **50** self-assembled into gels mostly in aromatic solvents, additionally one gel was formed in 1-octanol by compound **50**.[94] Compound **49** with cholyl backbone was only able to gelate chlorobenzene. On the contrary, compound **50** with dehydrocholyl backbone formed gels in a larger variety of solvents: gels in toluene, *p*-xylene and 1-octanol, and weak gels in cumene, mesitylene and ethylbenzene.



45 R^1 = OH, R^2 = H, R^3 = H
46 R^1 = OH, R^2 = α-OH, R^3 = H
47 R^1 = OH, R^2 = β-OH, R^3 = H
48 R^1 = OH, R^2 = H, R^3 =OH
49 R^1 = OH, R^2 = α-OH, R^3 = OH
50 R^1 = O, R^2 = O, R^3 = O

FIGURE 24 Bile acid derivatives **45–50** containing cysteamine side chain.

Compounds **51–54** with amidoalcohol moiety (Figure 25) were observed to form gels and weak gels in aromatic and chlorinated solvents.[95] Most of the gel systems were formed after cooling to room temperature, except the toluene and *p*-xylene gels of compound **51** which were formed after refrigeration overnight. All compounds **51–54** were poorly soluble or insoluble to solvents with low boiling points. The exception from this were methanol and chloroform.
Differences in gelation abilities were not clearly observed with regard of the steroidal backbone and the side chain in the case of compounds **51–54**. The lithocholyl backbone containing compounds **51** and **53** formed only one gel more than the deoxycholyl backbone containing compounds **52** and **54**. Differences regarding the consistence of the gel and the formation conditions were observed. In 1,1,1-trichloroethane compound **51** formed a gel whereas compound **52** formed a weak gel. Furthermore, in two solvents (toluene and p-xylene) the compound **51** formed a gel upon refrigeration, whereas compound **52** formed a gel at room temperature. The side chain influenced on the gelation as well: longer side chain of compounds **53** and **54** resulted in weaker gel-forming ability. For example, in chloroform all compounds **51–54** formed gels but compounds **53** and **54** formed weak gels. Additionally, in 1,2-dichloroethane and 1,1,1-trichloroethane only gelation of compounds **51** and **52** was observed.

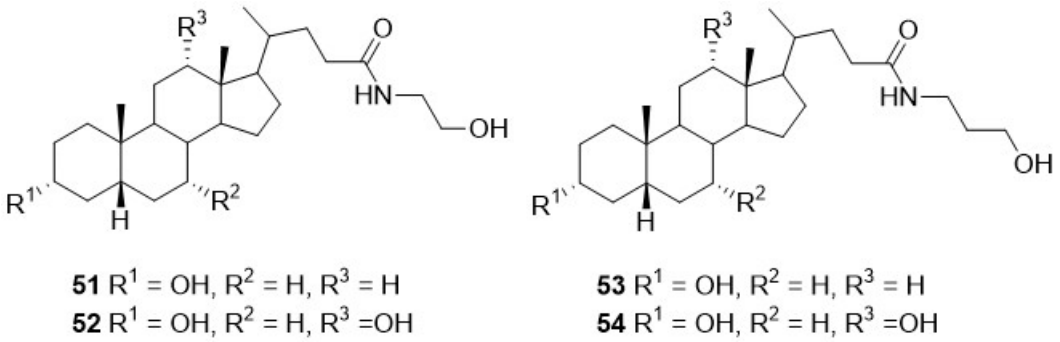


FIGURE 25 Bile acid derivatives **51–54** containing amidoalcohol moiety.

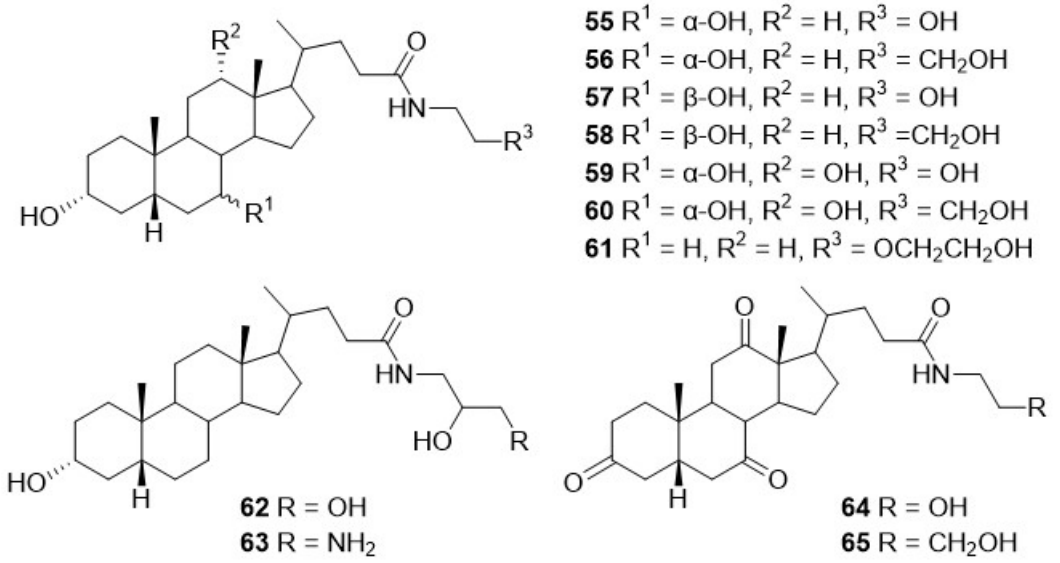


FIGURE 26 Bile acid derivatives **55–65** containing amidoalcohol moiety.

Valkonen *et al.* extended the study of amidoalcohols to compounds **55–65** (Figure 26).[96] Unfortunately, none of the compounds exhibited gel formation ability despite of the close structural similarity to compounds **51–54**. Since compounds **51–54** thickened acidic aqueous solvents, it was speculated that a very small amount of water could be required for gelation to occur by compounds **55–65**.

When comparing the structurally similar compounds **34–36** (with amine side chain),[93] compounds **45**, **46** and **48** (with thiol side chain)[94] and compounds **51**, **52** and **55** (with hydroxyl at the end of the side chain),[95,96] differences in the gelation ability are observed. When measured by the number of gels formed in each group of compounds, the compounds **51** and **52** are most effective gelators.

Within the series of compounds **34–36**[93] and compounds **45**, **46** and **48**[94], the trend for precipitation and solubility was similar: mainly precipitation from aromatic solvents and, in general, high solubility to non-aromatic solvents. Compounds **51** and **52**[95] follow similar trend, but gelation was observed in

aromatic solvent even though the compounds were partially soluble after heating. This demonstrates well the intricate balance between solubility and insolubility which is required for gel formation.

The lithocholyl backbone seems to promote the gelation process, as in all compound series compounds **34**, **45** and **51** form gels. The difference in gel forming ability between these three compounds lies in the side chain. Compound **34** with amine at the end of the side chain is slightly more efficient gelator (gel systems in three solvents) than compound **45** with thiol (one weak gel). The most effective gelator measured by the number of gels formed, is compound **51**, hence gelation property increases as **45>34>51**.

There is no clear correlation in gel forming ability regarding other steroidal backbones than lithocholyl and chenodeoxycholyl backbones. As mentioned, lithocholyl backbone promotes gelation. Instead, the chenodeoxycholyl backbone hinders the gel formation of multiple compounds (compounds **36**, **39**, **42**, **46** and **55**). Additionally, ursodeoxycholyl backbone does not promote gelation as can be observed from the properties of compounds **47** and **57**.

Multiple amino acid conjugates of bile acids have been observed to act as organogelators in addition to the previously discussed cysteamine derivatives **45–50**.[94] Two out of three L-methionine methyl ester conjugates of bile acids (Figure 27) were observed to form gels by Noponen *et al*.[97] As is usual for bile acid derivatives, the deoxycholyl compound **67** did not form any gels in the 26 solvents tested in the study. The lithocholyl derivative **66** formed majority of the gels (six in total) and cholyl derivative **68** formed four gels. Although the compound **66** formed more gels, compound **68** formed stronger and more transparent gels in the gelation tests carried out as 1 % (w/v). Toluene, benzene and *tert*-butylbenzene were the common solvents in which the gels were formed by compounds **66** and **68**.

Willemen *et al*. studied a large family of different cholic acid amides derivatised with amino acid esters (compounds **69–82** in Figure 28).[98] In general, gels formed in benzene and toluene were reported to be very stable, months at room temperature, transparent and mechanically stable. Additionally, all gels were thermoreversible. Compounds **71**, **81**, and **82** were not detected to form any gels, instead they preferred to crystallize.

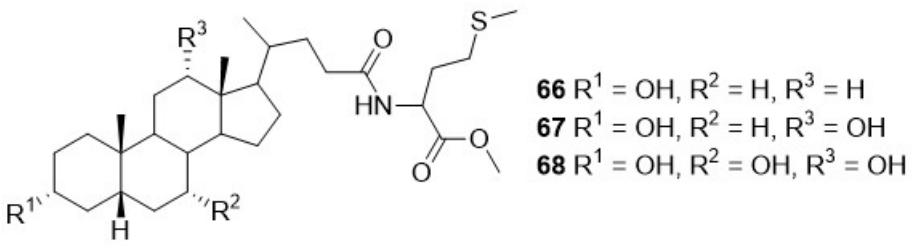


FIGURE 27 Three L-methionine methyl ester conjugates of bile acids.

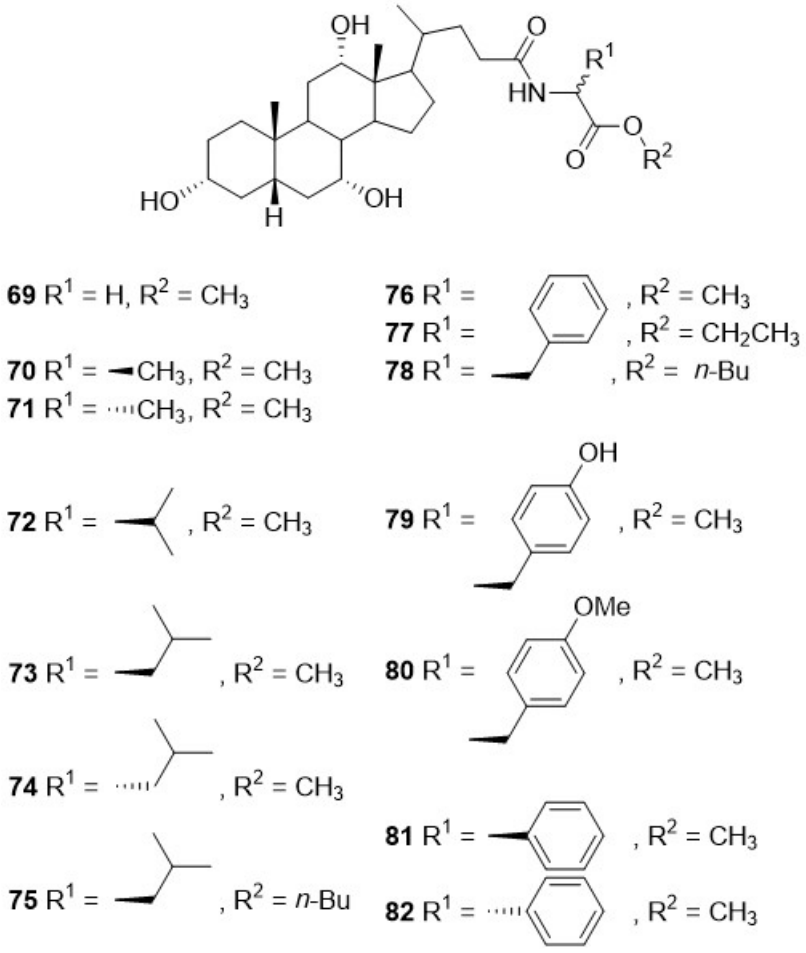


FIGURE 28 Divergent cholic acid derivatives studied by Willemen *et al*.

Compound **69** (with glycine methyl ester moiety) was not observed to gelate any solvents, it was mainly insoluble to the solvents tested. Four gels were formed by compound **72** with large *iso*-propyl group (namely L-valine methyl ester) at the side chain (compound **69** had hydrogen at the same position). The gels in benzene and toluene were stable, but gels in butylbenzene and cyclohexene disintegrated after one day. Larger amino acid moiety had an impact on the solubility in a manner which led to suitably soluble compound **72** forming gels.

The effect of L- and D-amino acid side chain was observed by comparing compounds **70** and **73** (L-alanine and L-leucine methyl esters, respectively) with compounds **71** and **74** (D-alanine and D-leucine methyl esters, respectively). Compound **71** was not soluble to any solvent, but compound **70** formed gels in benzene and toluene. This difference can be due to the better solubility of compound **70**. In the case of compounds **73** and **74**, the D-amino acid moiety led to more efficient gelation ability regarding two factors. Compound **74** formed more gels and the gel in benzene had higher melting point than the benzene gel of compound **73**.

The length of the alkyl chain at the amino acid moiety of the compounds had major effects on the stability of the gels. Three cholyl L-phenylalanine esters (compounds **76–78**) with different alkyl chain lengths formed weaker gels as the

chain length increased. Compound **76** with methyl group at the amino acid moiety self-assembled into gels in the same solvents as compound **72**. Compounds **77** (ethyl group) and **78** (*n*-butyl group) also formed gels in the same set of solvents, but the gels were not as stable. Gels produced by compound **77** were not stable in regard of time: gels started to collapse after one day, turning from transparent to opaque. When observing the melting point of the gels, the gels of compound **78** had 10 ˚C lower melting points. The same phenomenon was seen when comparing the compounds **73** and **75** with methyl and *n*-butyl group at the amino acid moiety, respectively.

The polarity of the amino acid moiety had an impact on the solubility and the gelation ability which was observed with compounds **79** and **80**. The L-tyrosine methyl ester was probably too polar which resulted in insolubility of the compound **79**. Instead, the methylated version (4-methoxyphenylalanine methyl ester) promoted gelation and compound **80** formed gels in benzene, toluene and butylbenzene. However, if the compound was too non-polar, no gelation was observed. This was evident with compounds **81** and **82** with L-phenylglycine and D-phenylglycine methyl ester moieties, respectively, which were observed to prefer crystallization.

The effect of the steroidal backbone was studied by preparing structurally similar L-leucine methyl ester derivatives in addition to compound **73**. Compounds **83–85** (Figure 29) with chenodeoxycholyl, deoxycholyl, and lithocholyl backbones, respectively, were not observed to form gels in any solvent tested. This led to the conclusion that three hydroxyl groups of the steroidal backbone were essential for gelation for bile acid derivatives. However, this hypothesis has been challenged in several studies[87,93–95,97,99] where cholic acid derivatives have not been the most effective gelator molecules when observing the gelation ability with respect of the steroidal backbone.

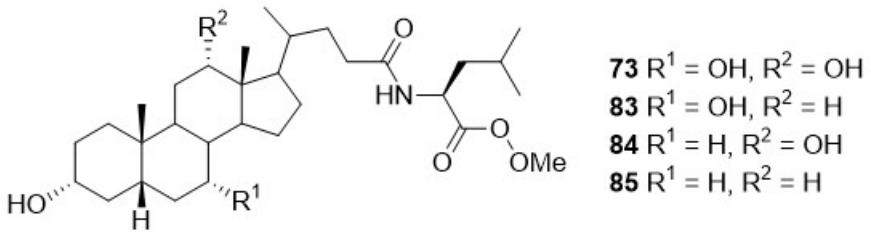


FIGURE 29 Analogues of compound **73** with different steroidal backbones.

When observing the gelation abilities of compounds **86–94** (Figure 30), significant differences in the gel formation was discovered depending on the gelators' structure.[99] From compounds **86–88**, containing the L-cysteine ethyl ester moieties, only cholyl derivative **88** formed gels. On the contrary, from compounds **89–91** with L-valine ethyl ester moiety and compounds **92–94** with L-serine methyl ester, only lithocholyl derivatives **89** and **92** were able to form gels.

The amino acid side chains had also an effect on the gelation properties in addition to the steroidal backbone. The side chains of compounds possessed

different polarity profiles, from relatively non-polar L-valine ethyl ester to moderately polar of L-cysteine ethyl ester and more polar L-serine methyl ester. The most polar compounds of this series, compounds **92–94** with L-serine methyl ester side chain, were not able to form any actual gels. Only two partial gels by compound **92** were observed in chlorobenzene and anisole. Compounds **89–91** with L-valine ethyl ester side chain were highly soluble to almost all of the 36 solvents tested, hence gel forming ability of the compound was low. Only compound **89** was observed to form a gel in tetrachloromethane after ultrasonic treatment. Moderate polarity of the side chain was the key to better gel forming ability: from compounds **86–88** with the L-cysteine ethyl ester side chain compound **88** formed five gels and five partial gels. The gels were formed in *o*-xylene, *tert*-butylbenzene, mesitylene, tetrachloromethane and diethyl ether. Partial gels of compound **88** were observed in *m*- and *p*-xylene, cumene and water.

Compound **88** was intriguing with regard of the process by which gels were formed. Gels in *o*-xylene, mesitylene and diethyl ether were formed by the conventional heating-cooling -cycle. However, gels in tetrachloromethane and *tert*-butylbenzene were formed after shaking the test tubes vigorously. This is quite unusual, because the samples were observed to precipitate after cooling but self-assembled into transparent gels after shaking. Also, in the case of *o*-xylene, mesitylene and diethyl ether gelation occurred even when the compound **88** was not fully soluble to the solvent in question.

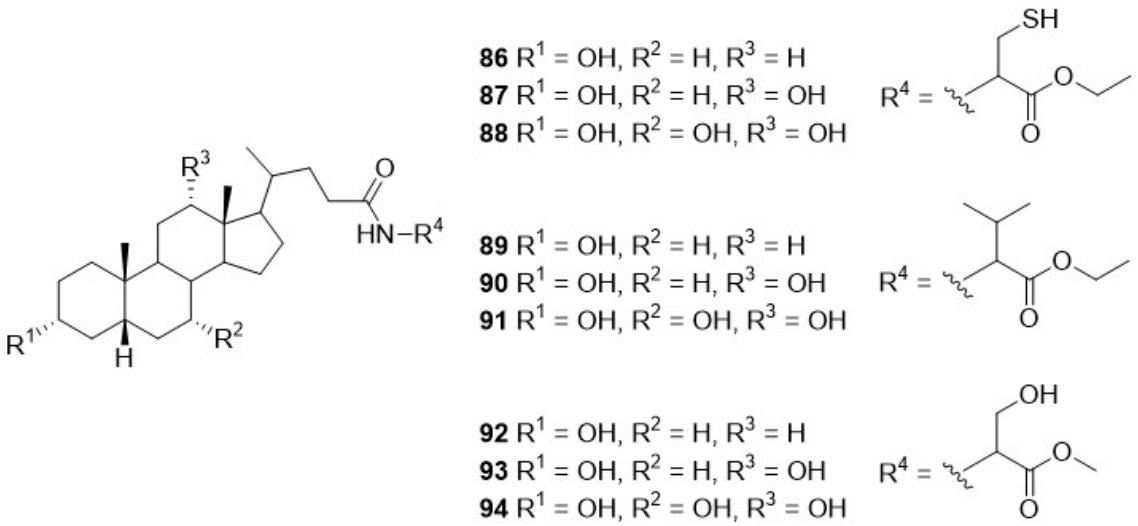


FIGURE 30 Bile acid amides with L-cysteine, L-valine and L-serine alkyl esters.

In addition to amino acid derivatives, Willemen *et al*. studied bile acid alkyl amides **95–105** (Figure 31) with respect of their gelation ability mainly in aromatic solvents as 1.5 % (w/v).[100] Cholyl derivatives **95–97** formed gels in cyclohexene in addition to aromatic solvents (benzene, toluene, ethylbenzene, styrene, chlorobenzene, and additionally in anisole in the case of compounds **96** and **97**). The length of the side chain did not have substantial effect on the gelation ability since compounds **96** and **97** formed gels only in one additional solvent when compared to compound **95**. The gels were observed to be thermoreversible and stable for a long period of time. Cyclohexene gels were exceptionally stable: the melting point was higher than the boiling point of pure cyclohexene. Interestingly, compound **100** with diacetylene moiety in the middle of the side chain gelated same solvents as compound **95**.

The addition of an aromatic moiety to the side chain in compounds **98** and **99** lowered the gel formation ability. Compounds **98** and **99** were observed to self-assemble into a gel after one day in cyclohexene. Compound **99** also formed gels in styrene (after one day) and benzene. The gels were also less stable when compared to compounds **95–97**.

When observing the change in gelation ability regarding the amide bond structure, some differences were detected. Compound **101** which has methyl group attached to the nitrogen of the amide bond was not able to form gels in the studied solvents. The 'free' amide bond is necessary for gel formation, since the structurally similar compound **96** with unhindered amide bond forms several gels. Compounds **104** and **105** containing reversed amide bond did not act as good gelators as corresponding compounds **96** and **97**. Compound **104** formed three gels when the corresponding compound **96** formed seven gels. Instead, compounds **97** and **105** formed gels in same solvents. The differences were in the gel formation time, gels in styrene and anisole by compound **105** were formed slower than in the case of compound **97**. When taking into account the gel melting points of benzene gels of compounds **96**, **97**, **104** and **105**, the gels of compounds **104** and **105** had 15 ˚C lower melting points.

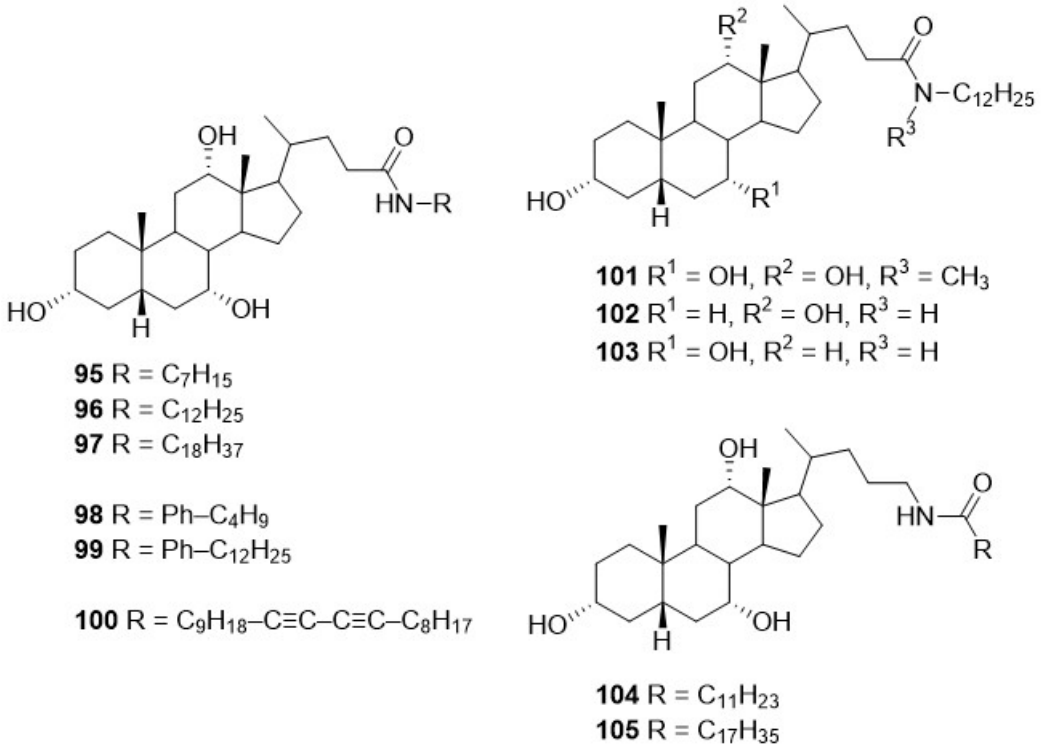


FIGURE 31 Bile acid alkyl derivatives.

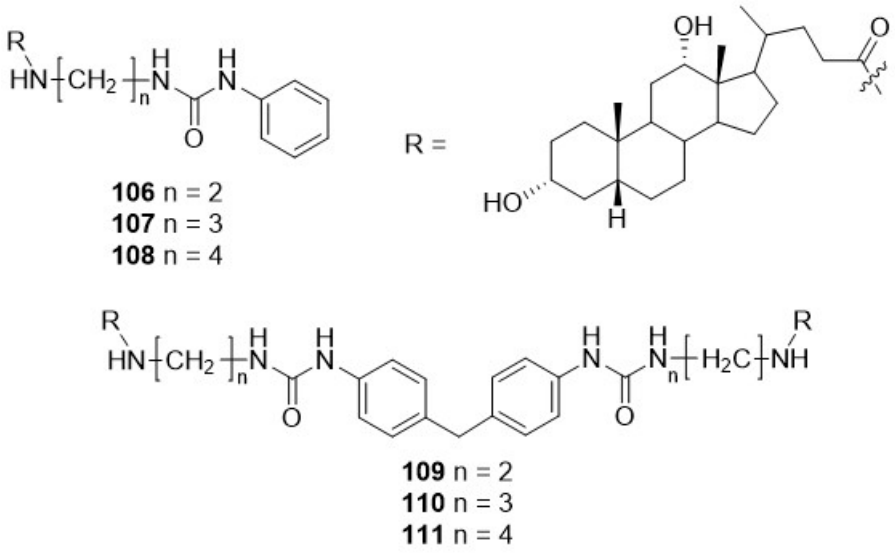


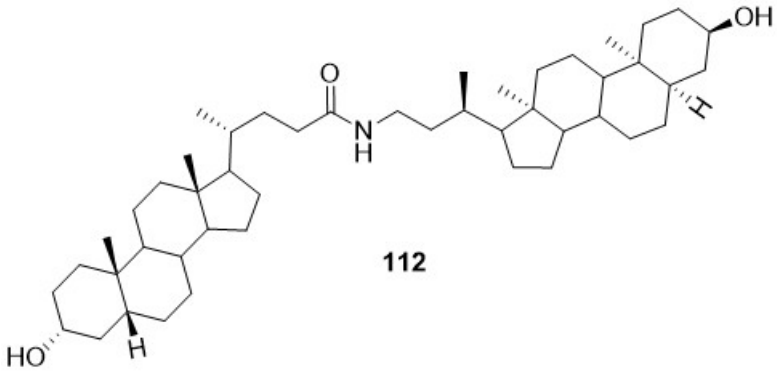
FIGURE 32 Deoxycholic acid alkylamide-phenylurea derivatives.

The bile acid core has an impact on the gelation ability when comparing compounds **96**, **102**, and **103**. As mentioned previously in the case of bile acid amino alkylamides[93] and cysteamine amides[94], in this case also the chenodeoxycholyl backbone prevents gel formation (compound **103**). However, compound **102** with deoxycholyl backbone formed a single gel cyclohexene.

Monomeric and dimeric deoxycholic acid alkylamide-phenylurea compounds **106–111** (Figure 32) were observed to have divergent solubility and

gel formation properties.[101] In addition to the effect of monomeric or dimeric nature of the compounds in question, the length of the alkyl linker chain had an impact to gel formation. The monomeric compounds **106–108** were observed to be more soluble to the 16 solvents studied than their dimeric counterparts **109–111**. Regarding the length of the alkyl chain connecting the steroidal and urea moieties, the solubility decreased in the order ethyl (compound **106**) > propyl (compound **107**) > butyl (compound **108**). For dimeric compounds **109–111** the order was different: propyl (compound **110**) > ethyl (compound **109**) > butyl (compound **111**).

Two compounds formed gels: monomeric compound **107** with propyl chain in chlorinated solvents and dimeric compound **111** with butyl chain in non-aromatic solvents. Compound **107** formed gel in chloroform and chlorobenzene, to which compound **111** was insoluble to. Instead, compound **111** self-assembled into gels in THF and 1-alcohols containing 7–10 carbons. The number of carbons in the alcohol was a relevant factor for the gelation as in alcohols with shorter alkyl chain from ethanol to 1-hexanol no gelation was observed.



112

FIGURE 33 Dimeric lithocholyl derivative.

Another dimeric bile acid based organogelator is compound **112** (Figure 33).[102] Interestingly, compound **112** containing two lithocholyl moieties did not gelate aromatic solvents as 1 % (w/v), such as toluene or *m*-xylene. Neither was gelation observed in alcohols, tetracholomethane, acetonitrile, and THF among other solvents. Compound **112** formed gels in three solvents: 1,4-dioxane, acetone and DMSO. The melting temperature of the DMSO gel was 52 ˚C and increased with increasing the amount of compound **112**. The DMSO gel of compound **112** was further utilized as an embedment material for photoluminescent CdS nanoparticles.

Dimeric cholyl derivatives **113–115** (Figure 34) were not efficient gelators, only three gels in total were formed in eleven solvents tested.[100] Compound **114** with long alkyl chain linker between the cholyl moieties did not act as a gelator. Shorter alkyl linker resulted in two gels in acetophenone and chloroform. The gels were whether as stable or transparent as the gels formed by compounds **95–97**, **100**, and **104**. Instead, the transparent gel in acetophenone by compound **115** exhibited similar melting point as benzene gels of compounds **95–97**.

Compounds **113–115** are more polar when compared to non-dimeric similar compounds due to the two cholyl moieties. Hence, it was speculated that compounds **113–115** require more polar solvents for gelation to occur. Similar effect was observed with compounds **107** and **111**: more polar dimer formed gels in more polar solvents.[101]

Deoxycholic conjugates of α-cyanostilbenes with large side chain were observed to gelate several solvents (Figure 35).[103] Compounds **116–119** formed gels mainly in aromatic and *n*-alkyl solvents and the minimum gelation concentration varied with respect of the compound and solvent in question. The alkyl chain length at the α-cyanostilbene moiety had an interesting effect on the gelation and gel properties.

Compounds **118** and **119** with longer alkyl chains self-assembled mainly into weak gels but gel systems were formed in a larger number of solvents than in the case of compounds **116** and **117**. Gels were formed at similar minimum gelation concentration (mainly from 1 % (w/v) to 9 % (w/v)) by all compounds **116–119** in aromatic solvents as well as in *n*-alkyl solvents. The minimum gelation concentration was significantly higher in DMF, DMSO and alcohols, such as decanol, for compounds **118** and **119**: from 8 % (w/v) to 16 % (w/v).

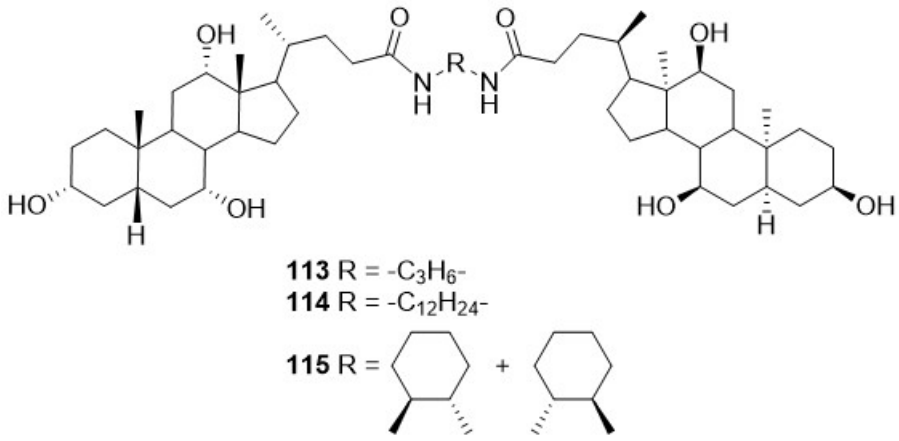


FIGURE 34 Dimeric cholyl derivatives.

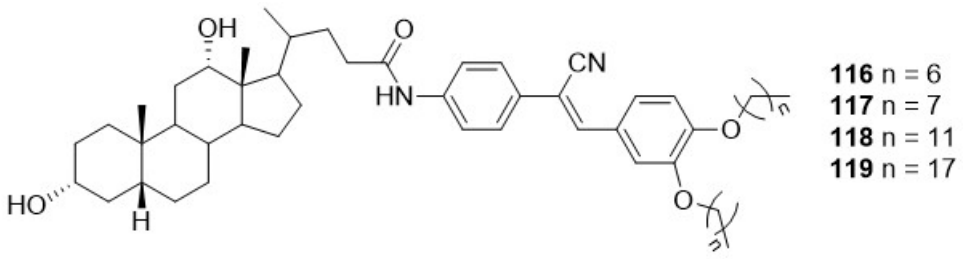


FIGURE 35 Deoxycholic acid appended with α-cyanostilbenes.

More stable gels were formed by compounds **116** and **117** with shorter alkyl chains in the α-cyanostilbene moiety, the only weak gels formed in hexane and heptane. The minimum gelation concentration was also higher in the before mentioned two solvents (10–12 % (w/v)). Gelation in longer *n*-alkyl solvents also required higher concentration of compounds **116** and **117**, varying from 7 % (w/v) to 9 % (w/v). Similar trend with respect of the solvent type was observed for compounds **118** and **119** as well. However, gelation in aromatic solvents was clearly more efficient for all compounds in the study as gelation occurred in concentrations varying in the range of 1–1.4 % (w/v). In Figure 36 seven yellow gels formed in divergent solvents by compounds **117** and **118** are shown.

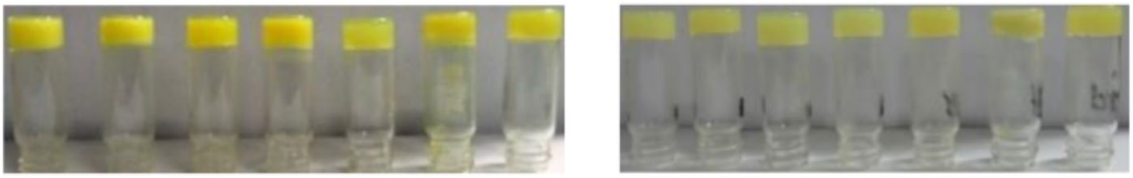


FIGURE 36 Yellow gels formed by compounds **117** (left) and **118** (right) in divergent solvents (from left to right: xylene, toluene, mesitylene, decane, dodecane, tetradecane, and hexadecane). Reprinted from Steroids, 160, D.S. Agarwal, R.P. Singh, P.N. Jha, and R. Sakhuja, Fabrication of deoxycholic acid tethered α-cyanostilbenes as smart low molecular weight gelators and AIEE probes for bio-imaging, 108659, Copyright (2020), with permission from Elsevier.

Steroidal backbone influences the gel forming properties, and on some occasions also to the properties of the gels. Sometimes the effect is not as clear and the correlation between certain steroidal backbone and the gel formation ability of the compound depend on the side chain, or the linker between two bile acid moieties. In general, lithocholyl and cholyl backbones promote gelation.[93–95,97,99,100,102] In some cases, also deoxycholyl and dehydrocholyl backbone.[93–95,100,101,103] When concerning organogelation, chenodeoxycholyl and ursodeoxycholyl backbones hinder gel formation.[93,94,96,98,100]

Length and nature of the side chain or the linking chain impacts on the gelation ability of the discussed compounds. Aminoalkyl amides[93] were more effective gelators when compared to the corresponding cysteamines[94] and amidoalcohols.[95,96] The answer to these differences may lie in the nature of the side chain. Polarity of the side chain is different, hydroxyl side chain is the most polar, amine is less polar, and thiol is moderately polar. This leads to the conclusion, that suitable polarity of the side chain is required for the compound to form appropriate interactions to self-assemble to a gel. Furthermore, similar effect of the mid-range polarity of the side chain can be seen in the case of amino acid derivatives of bile acids. When the amino acid moiety possesses moderate polarity, better gel forming ability was observed.[98,99] However, the effect of the length of the alkyl chain either in between the amide bond and the functional group at the end of the chain or at the ester of the amino acid moiety was unclear. Both deterioration and enhancement of the gel forming ability as the length of the alkyl chain increased. When considering the organogelation of the amino acid

derivatives of bile acids, the D-form was more effective in one study.[98] Additionally, reversed amide bond reduces the compounds' ability to form gels, as was discovered by Willemen *et al.*[100] The importance of the 'free' amide bond was also observed in the same study: if the hydrogen was replaced by a methyl group no gelation was detected.

The most common solvents promoting gel formation of bile acid derivatives are aromatic. Gelation was observed also in divergent chlorinated solvents from chlorobenzene to tetrachloromethane. Additionally, in few cases gelation occurred in alcohols, and other polar solvents. Global and multi-term solvent parameters can be utilized in the search for suitable solvents for gelation.[104] On the other hand, the effect of the solvent to gelation can be evaluated using the same parameters, as was done by Löfman *et al.* by Kamlet-Taft analysis.[93,105] Three parameters are used in Kamlet-Taft analysis: the α-parameter (hydrogen bond donor ability), β-parameter (hydrogen bond acceptor ability), and π*-parameter (polarizability). It was observed that the common factors of the gel forming solvents were the lack of hydrogen bond donor ability and moderate values of hydrogen bond acceptor ability which is the case with aromatic solvents generally.[93] If the ability of the solvent to act as hydrogen bond acceptor was too high, disrupting the gelation process, no gelation was observed even though the hydrogen bond donor ability was zero. Instead, there were no clear correlations among the π*-values of the solvents. When considering the gelating chlorinated solvents, there seems to be a delicate balance between the Kamlet-Taft parameters as no clear trend was observed. As a conclusion, aromatic solvents are good gel forming solvents due to their suitable hydrogen bond donor and acceptor profile. With other gelation inducing solvents the Kamlet-Taft parameters form an extremely fine balance leading to appropriate interactions between the gelator molecules and the solvent and gelator molecules.

2 RESULTS AND DISCUSSION

2.1 Aims and background of the study

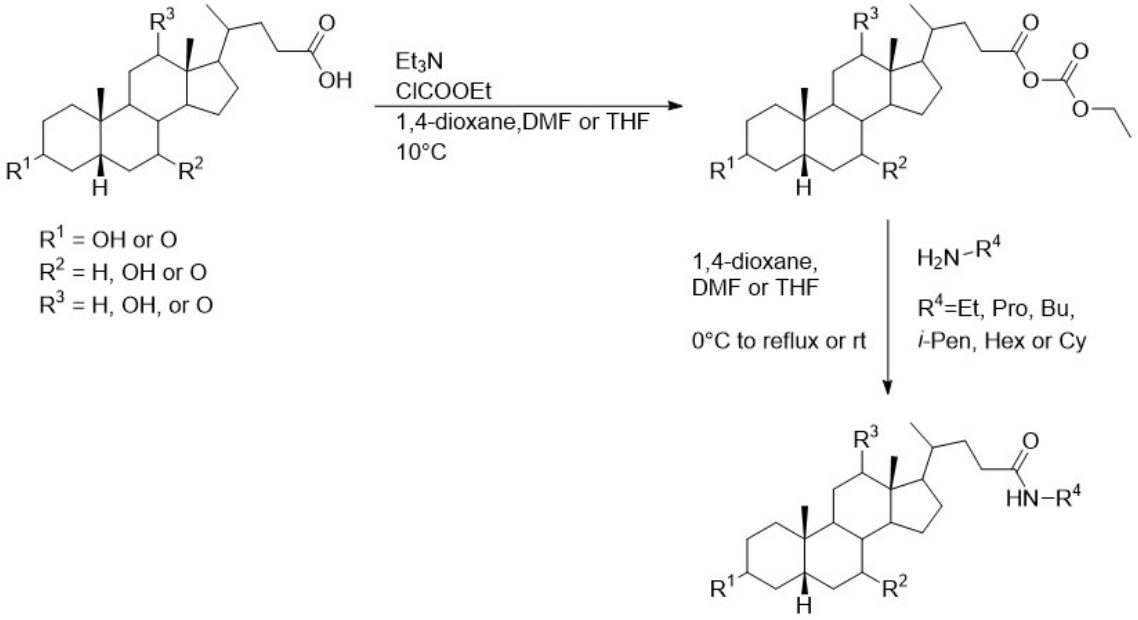
Bile acids, especially their derivatives, are remarkably versatile materials for research. Because of their availability, biocompatibility, enantiomeric purity, cost effectivity, and fascinating properties, bile acids have been studied in detail in many fields of science. In addition, bile acids can be modified with relative ease in multiple ways. In our research group, divergent bile acid amides have been studied in the past with respect of the bile acid derivatives ability to form gels in different solvent systems and even with metal ions.[87,93–97,99,101,106] Since the bile acid amide synthesis is well known, and provides quickly multiple molecules for research, we decided to utilize the same synthesis route to create a family of bile acid derivatives containing simple alkyl chains with divergent lengths. We were fascinated to study the impact of the side chain attached to the bile acid core on to the self-assembly of gel systems with regard of the length of the side chain.

In the beginning, bile acid amides with ethyl chain were studied.[I] After that, derivatives with propyl, butyl and *iso*-pentyl chain provided further insight to the gel formation abilities of simple alkyl chain amides.[II] The branched *iso*-pentyl derivative led the research into new direction when the question of the effect of circular alkyl side chain arose. Thus, the last step of this thesis work was to investigate the properties of bile acid amides with hexyl and cyclohexyl side chains.[III]

The synthetized compounds were characterized with NMR spectroscopy, mass spectrometry and their solid-state properties were studied with single crystal X-ray diffraction. Solid state NMR was utilized in the study of both the crystallized compounds and some of the gels formed. Scanning electron microscopy (SEM) was used to visualize the formed gel systems. The gel forming properties of the compounds were studied in detail in different solvents and solvent mixtures.

2.2 Synthesis of bile acid derivatives[I–III]

The bile acid alkyl amide derivatives studied were synthetized using the mixed anhydride method, which is well known in the literature[87,94,97,99,106–109], to obtain the desired alkyl derivatives. The synthesis was relatively uncomplicated, but the purification process of the products was time consuming. The conditions of the reactions varied slightly, as described in the articles I–III. As presented in Scheme 1, the mixed anhydride was prepared from bile acid and ethyl chloroformate. This intermediate product was not isolated. Amine was added to the solution containing the mixed anhydride and the amide bond formation occurred. The raw products were purified with either column chromatography or precipitation from water, followed by re-crystallization. Unoptimized yields of the pure compounds varied from 11 % to 75 %.



SCHEME 1 General procedure for the synthesis of the bile acid alkyl amide derivatives.

In this work, 19 compounds were prepared in total. The author is most indebted for students, who have helped in the synthetic work in times she was unable to work in the laboratory. All the compounds prepared are presented in Figure 37.

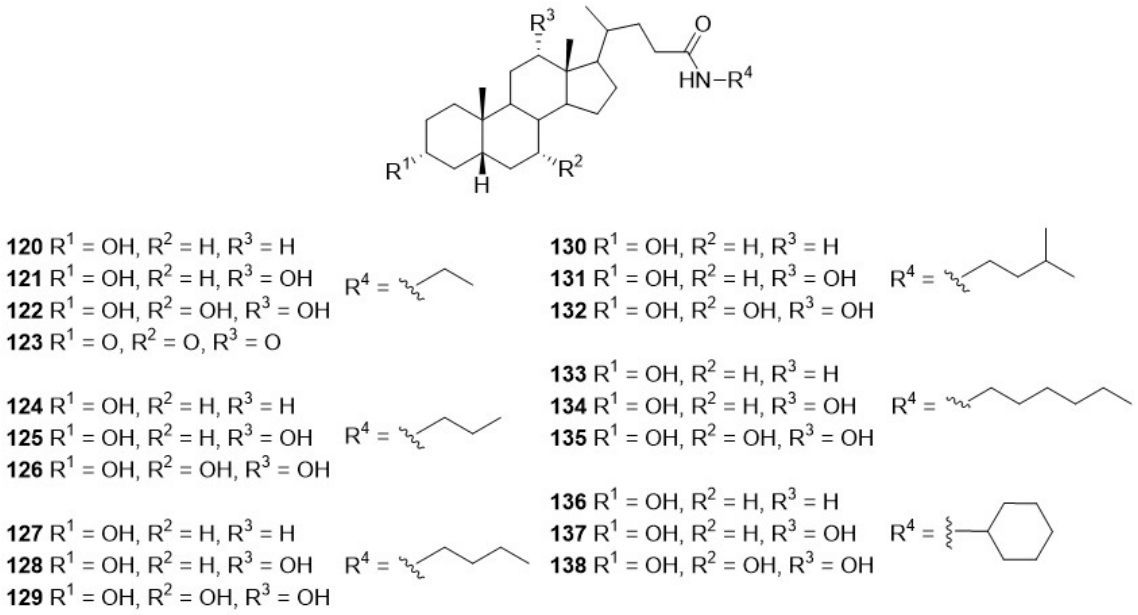


FIGURE 37 Compounds prepared during the thesis work.

2.3 Self-assembly studies of bile acid alkyl amides[I–III]

Self-assembly properties of the compounds **120–138** were tested in various divergent types of solvents from aromatic solvents to alcohols and water. In addition, the self-assembly ability of compounds **120–129** were studied in aqueous solvent mixtures. Majority of the gel systems were formed by lithocholyl derivatives. Some gel systems were observed to be formed by cholyl or dehydrocholyl derivatives. The only dehydrocholyl derivative (compound **123**) studied did not form any gels in single solvent tests, which is why dehydrocholic acid was excluded from further studies.

As is common for bile acid derivatives, most of the gel systems observed were formed in aromatic solvents such as benzene, toluene, and *p*-xylene. Organogelation was observed to occur by multiple compounds. In addition to aromatic solvents, gelation occurred for example in acetone, acetonitrile, and ethyl acetate. In total, 101 divergent gel systems were observed to form in the gelation tests with single solvents. When concerning the gel forming ability of the compounds with regard of the steroidal backbone, the greatest number of gels were assembled by lithocholyl derivatives (compounds **120**, **124**, **127**, **130**, **133** and **136**), 69 different gel systems in total. Cholyl derivatives (compounds **126**, **129**, **132**, **135** and **138**) were responsible for the second largest number of gel systems (30 gel systems). Deoxycholyl derivative **134** self-assembled into a gel and a partial gel (2 gel systems) which is very fascinating, since all the other deoxycholyl derivatives were either extremely soluble to or precipitated out from all of the solvents tested.

Benzene and other aromatic solvents are generally good solvents regarding gelation, because they are flat and small. Which means that they fit perfectly into the cavities in between the bile acid derivatives without disturbing the self-assembly of the compounds into gel network. No gelation was observed in alcohols or chlorinated solvents, excluding chlorobenzene and carbon tetrachloride. This is due to the high solubility of all the compounds studied to chloroform, DCM, and alcohols. Additionally, water was a poor gelating solvent because of the restricted solubility of bile acids in water. However, hydrogelation of compounds **120** and **123** was observed. The lithocholyl derivative **120** formed five hydrogel systems of which three were strong gels. Two of the strong gels formed as 2 % (w/v) in 50:50 H_2O:2-propanol and 10:90 H_2O:ACN. One strong gel was observed as 1 % (w/v) in 70:30 H_2O:MeOH. All strong hydrogels were thixotropic by nature, meaning they collapsed immediately when exposed to mechanical stimuli (disturbed with a spatula). Compound **120** also gave rise to one weak hydrogel (1 % (w/v) in 10:90 H_2O:acetone) and one partial hydrogel (2 % (w/v) in 10:90 H_2O:acetone). The dehydrocholyl derivative **123** formed one partial hydrogel in 70:30 H_2O:MeOH as 2 % (w/v). Compounds **121**, **122** and **124–129** were also studied with regard of the ability to form hydrogels in divergent solvent systems, but no hydrogelation was detected. This is probably due to the more lipophilic nature of the compounds with larger side chains.

As mentioned, most of the deoxycholyl derivatives (**121**, **125**, **128**, **131** and **137**) did not show any gel forming ability. This is a common phenomenon concerning deoxycholyl derivatives, which has been reported several times[93–100] and it is thought to be due to the intermediate solubility profile of deoxycholic acid. Hence, it was surprising to observe deoxycholyl derivative **134** self-assembled to either a partial gel or a gel. When examining the gelation ability of deoxycholyl derivatives with regard of the side chain, the most lipophilic compound containing linear hexyl side chain is the best gelator.

The effect of the side chain onto the self-assembly properties was enchanting to study. Few overall effects were observed in the series of compounds presented in this thesis. First, the number of gel systems formed increased in the series from ethyl derivatives to hexyl derivatives. Ethyl and propyl derivatives (compounds **120–123** and **124–126**, respectively) formed 11 gel systems each, 13 were formed by butyl derivatives (compounds **127–129**), 22 by *iso*-pentyl and 23 by hexyl derivatives (compounds **130–132** and **133–135**, respectively). There was a small drop in the total number of formed gel systems, when comparing the hexyl and cyclohexyl derivatives (21 gel systems by compounds **136–138**). But, in the total amount of divergent solvents promoting gelation the cyclohexyl derivatives gelated more solvents than the butyl derivatives and less than *iso*-pentyl derivatives. Additionally, in the case of ethyl derivatives only lithocholyl derivative **120** formed gels. Compared to the other side chains, also the cholyl derivatives self-assembled into gel systems in addition to the corresponding lithocholyl derivatives.

Even number of carbons in the side chain had also impacted on the self-assembly properties regarding the lithocholyl backbone containing molecules.

More good gels than weak or partial gels was formed by ethyl and butyl derivatives, compounds **120** and **127**, respectively. The hexyl derivative (**133**) formed almost equal amounts of good gels (7 in total) and partial gels (8 in total). Uneven numbered side chains (propyl and *iso*-pentyl derivatives, compounds **124** and **130**, respectively) formed notably larger amounts of either weak or partial gels than good gels.

On the contrary, the effect of even and odd numbered side chains had an opposite outcome when looking into the cholyl backbone containing compounds. The self-assembly was significantly weaker in even numbered side chain compounds **122** and **129**. The ethyl derivative **122** did not form any gel systems and butyl derivative **129** formed only two gels, one good and one weak. Hexyl side chain was an exception in this case also, as only good gels were formed by the compound **135**. In the series of the odd numbered side chain, it is evident that the branched *iso*-pentyl side chain of compound **132** was promoting the self-assembly more than the linear propyl side chain in compound **126**. Compound **126** formed only one good gel and three weaker gel systems, whereas compound **132** formed six good gels and four weaker gel systems.

Intriguing effect arose when observing the differences in between the hexyl and cyclohexyl side chain regarding the lithocholyl and cholyl backbones. In the case of hexyl side chain, the effect of bile acid backbone is clear. Although the lithocholyl derivative **133** formed more gel systems by numbers, the cholyl derivative **135** formed exclusively good gels. This might be due to the compound **135** ability to form hydrogen bonded polymers, as were seen in the single crystal structure of the compound. When studying the cyclohexyl derivatives **136** and **138**, the effect was the same, although not perhaps as distinct. The ratio of good gels vs partial gels formed was 1:12 with compound **136** and 1:2 with compound **138**. The effect of solvent to gelation ability of compounds **133** and **136** with lithocholyl backbone was complementary to the structure of the side chain: hexyl side chain derivative (**133**) formed a gel in *n*-hexane and cyclohexyl side chain derivative (**136**) in cyclohexyl. When the solvents were the other way around, both compounds formed partial gels. Hence, there is a pronounced correlation between the structure of the compound and solvent. In addition, when comparing the compounds **133** and **136** in other solvents, it was seen that when compound **133** formed a good gel, compound **136** formed a partial gel (excluding cumene in which both compounds formed partial gels).

Mechanical stability of the gels was observed qualitatively by exposing the gel sample to mechanical stimuli (disturbing with a spatula). It was observed that the mechanical stability increased with the increasing length of the side chain. The organo- and hydrogels of ethyl derivatives **120** and **123** disintegrated immediately when the spatula touched the surface of the gel. On the contrary, gels formed by hexyl- or cyclohexyl derivatives were significantly more stable. The gel either held its shape after a portion of it was removed with a spatula or returned to an even-surfaced gel. Some of the gels were like food jelly and some stretched to a great extent. For example, the benzene gel of compound **135** was difficult to remove from the test vial to get a SEM-sample. And after removing

the gel, it was sticky and even harder to place on the sample stubs carbon tape. Furthermore, the temporal stability followed same pattern. Even the disturbed gels of hexyl- and cyclohexyl gels were stable for over six months. But the gels of ethyl derivatives started to collapse after few days. The propyl, butyl, and *iso*-pentyl derivatives had intermediate stability. Some of the gels did not collapse immediately when disturbed with a spatula but started to disintegrate after few hours. When the gels were left undisturbed, they were stable for few months.

Willemen *et al.* suggested two decades ago that the amount of hydroxyl groups in the steroidal backbone is essential for gel formation.[98] In the study, only cholyl derivatives were observed to form gels whereas corresponding compounds with different steroidal backbone did not act as gelators. The hypothesis that only cholyl derivatives are able to form gels has been challenged many times since[87,93–95,97,99,101,106] and in the current thesis. The number on hydroxyl groups in the steroidal backbone has indeed an effect to the gelation ability of the compounds, but it seems that rather two hydroxyl groups than three in the steroidal backbone prevents gelation. As discussed in the chapter 1.4, deoxycholyl and chenodeoxycholyl backbones tend to hinder gelation in addition to dehydrocholyl backbone.

Kamlet-Taft solvent parameters[105] were used to examine the solvents promoting and hindering gelation in the case of compounds **133–138**. Kamlet-Taft parameters include three parameters that can be utilized in the analysis of solvent interactions in gel systems. Two of the parameters describe the hydrogen bonding ability of the solvent: α-parameter and the β-parameter describe the hydrogen bond donating and accepting ability of the solvent, respectively. The third parameter, π*-parameter, refers to the polarizability of the solvent. Previously[93], the α-parameter was observed to be the most important Kamlet-Taft parameter effecting on the immobilization of the solvent by a gelating compound. When looking into the gelating solvents of compounds **133–138**, the situation is the same as the α-parameter is zero for nearly all the gel-forming solvents. Exceptions to this were acetonitrile (0.19), formamide (0.71), and ethylene glycol (0.90).

Additionally, the majority of the β-parameter values (hydrogen bond acceptor ability) were in accordance with previous studies of bile acid derivatives[93], with moderate values from 0.10 to 0.31. Some of the gelating solvents (diethyl ether, ethyl acetate, dimethyl sulfoxide, and ethylene glycol) have higher values of β-parameter (0.47, 0,45,0.76, and 0.52, respectively). In the previous study[93] the higher β-value was concluded to prevent the gelation process. However, with compounds **133–138** similar correlation was not observed. This could be due to the differing structures of the gelating molecules from two points of view. First, the molecule may be able to form gels in larger versatility of solvents when there is no functional group at the end of the aliphatic side chain. Second, the steroidal backbone. In the current thesis lithocholyl, deoxycholyl, and cholyl backbones were used; whereas in the previous study[93] instead of cholyl backbone, chenodeoxycholyl backbone was used.

For most of the gelating solvents the value of the π*-parameter varies between 0.10 and 0.56. However, acetonitrile, formamide, and ethylene glycol stand out with significantly higher values in addition to chlorobenzene and anisole.

When looking into the Kamlet-Taft parameters of the chlorinated solvents, an interesting difference is observed. Gel formation inducing chlorobenzene is clearly different from chloroform and dichloromethane, which did not induce gel formation. Chloroform and dichloromethane both possess slightly more hydrogen bond donor ability (α-parameter) than chlorobenzene. Instead, the hydrogen bond acceptor ability (β-parameter) appears to be a more significant factor for gelation within chlorinated solvents, since chlorobenzene has the highest value of the three solvents compared. Similar observation applies to the polarizability of the solvent (π*-parameter), which leads to the conclusion that the solvation of peripheral groups of the gelator molecule and the fiber-fiber interactions play a major role in gel formation in chlorobenzene.

Anisole seems to stand out from the other aromatic solvents at first glance when comparing the aromatic solvents promoting gel formation (excluding chlorobenzene). Anisole has markedly higher β- and π*-values than other the aromatic solvents, the α-value being zero as is the case with all the other aromatic solvents. However, when the ratio of β/π* is calculated, all aromatic solvents have similar values (around 0.30).

Compounds **133–138** were highly soluble in alcohols, hence gelation was not observed. Generally, all Kamlet-Taft parameters were in the same range for the alcohols. This leads to a hypothesis that solvents which possess relatively equal hydrogen bond donor and acceptor abilities are not appropriate solvents for gelation of bile acid amides.

2.4 Morphology of the gel systems[I–III]

Scanning electron microscope images taken from the dried samples of compounds **120–138** revealed both fibrous and spherical structures. In some cases, the fibres and spherical structures co-existed. Fibres were flat, bar-like or hollow bars. Some of the compounds, which formed fibrous gel networks, the gel fibres consisted of smaller fibres. In the gel systems which constituted of spherical shapes, the spherical assemblies were either elided or more separate particles.

Interestingly, it was observed that lithocholyl and deoxycholyl derivatives showed fibrous nature in the SEM images. Instead, the cholyl derivatives formed mainly spherical shapes. The explanation to this phenomenon can be due to the divergent nature of the steroidal backbones, as similar phenomenon was observed previously.[99] On the other hand, the differences in the appearance of the gel network may not arise solely from the steroidal backbone because in the past lithocholyl derivatives have formed spherical assemblies as well as fibrous networks.[93]

In the case of compound **120**, with ethyl side chain, half of the SEM samples were prepared by the traditional method by pipetting hot solution of the gelator in appropriate solvent or solvent mixture to the sample stub. The other half of hydrogel samples were prepared by scooping a self-assembled gel from the test tube to a sample stub. The solvents and solvent mixtures used were the same in both methods of sample preparation. There were differences in the structures observed in the SEM images depending on the sample preparation method, as was most clearly observed in the 2 % (w/v) hydrogel in 50:50 H_2O:2-propanol. In the samples prepared from the hydrogel, spherical assemblies consisting of fan-shaped bundles of fibres were seen (Figure 38 a). On the contrary, SEM images revealed fibrous assemblies resembling stalks of grass in the samples prepared with the traditional method (Figure 38 b). In all the hydrogels studied the width of the fibres was similar (1–15 μm) but the length varied. Single fibres produced by the traditional sample preparation were 20–240 μm long and longer in the scooped samples (60–410 μm). The diameter of the spherical assemblies formed by fibres varied from 390 μm to 740 μm. The reason for the differences of the structures observed may lay in the self-assembly time: the pipetted samples formed a gel instantly whereas the scooped samples were allowed to self-assemble over an hour in a test tube.

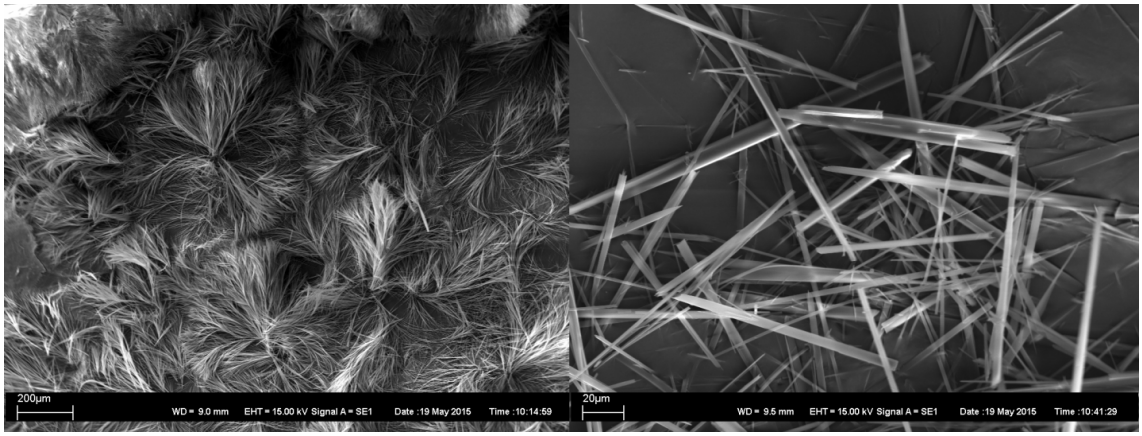


FIGURE 38 2 % (w/v) gel in 50:50 H_2O:2-propanol of compound **120**. Sample prepared from gel (left) and sample prepared from hot solution (right).

The SEM samples for all the other compounds were prepared by the scooping method. When compared to the ethyl side chain derivative **120**, the lithocholyl derivatives **124** and **127** (with propyl and butyl side chains, respectively) formed significantly smaller flat fibres (Figure 39 a and b, respectively). The fibres in the chlorobenzene gels were similar in size for compounds **124** and **127**, width of 600–800 nm and length 40–60 μm. As 2 % (w/v) the fibres formed fan-shaped bundles which were not arranged into spherical assemblies (Figure 39 a and b, respectively) similarly to the ethyl side chain derivative **120**. When observing the gel network formed by compound **130** (*iso*-pentyl side chain) in chlorobenzene, the fibres were bigger: up to 1.6 μm wide and approximately 200 μm long (Figure

39 c). Lithocholyl derivatives with hexyl and cyclohexyl side chains (compounds **133** and **136**, respectively) formed a fibrous gel network consisting of uniform-looking fibres (Figure 40 a and c). The width and length of the fibres varied. The fibres formed by compound **136** were versatile in length (from 61 µm to 775 µm), less so in the case of compound **133** (from 19 µm to 376 µm). When comparing the width of the fibres, compound **136** possessed the wider fibres (from 1.5 µm to 7.8 µm) and compound **133** (from 654 nm to 6.6 µm) thinner ones. The texture of the gels formed in benzene and toluene was different. The gel formed by compound **136** in benzene was clearly fibrous (also to the unaided eye), whereas the gel of **133** in benzene was more homogenous and cleavable.

The lithocholyl derivatives **124** and **130**, containing odd numbered side chains, were detected to form hollow nanotubes that co-existed with fibres in ethyl acetate (Figure 41). Compound **124** with propyl side chain formed bigger nanotubes than compound **130** with *iso*-pentyl side chain. The width of the tubes was 30 µm or 700 nm to 3.3 µm, and length 390 µm or 12–32 µm, respectively. The flat fibres formed by compound **124** were little bigger than in chlorobenzene (width 950 nm and length 60 µm).

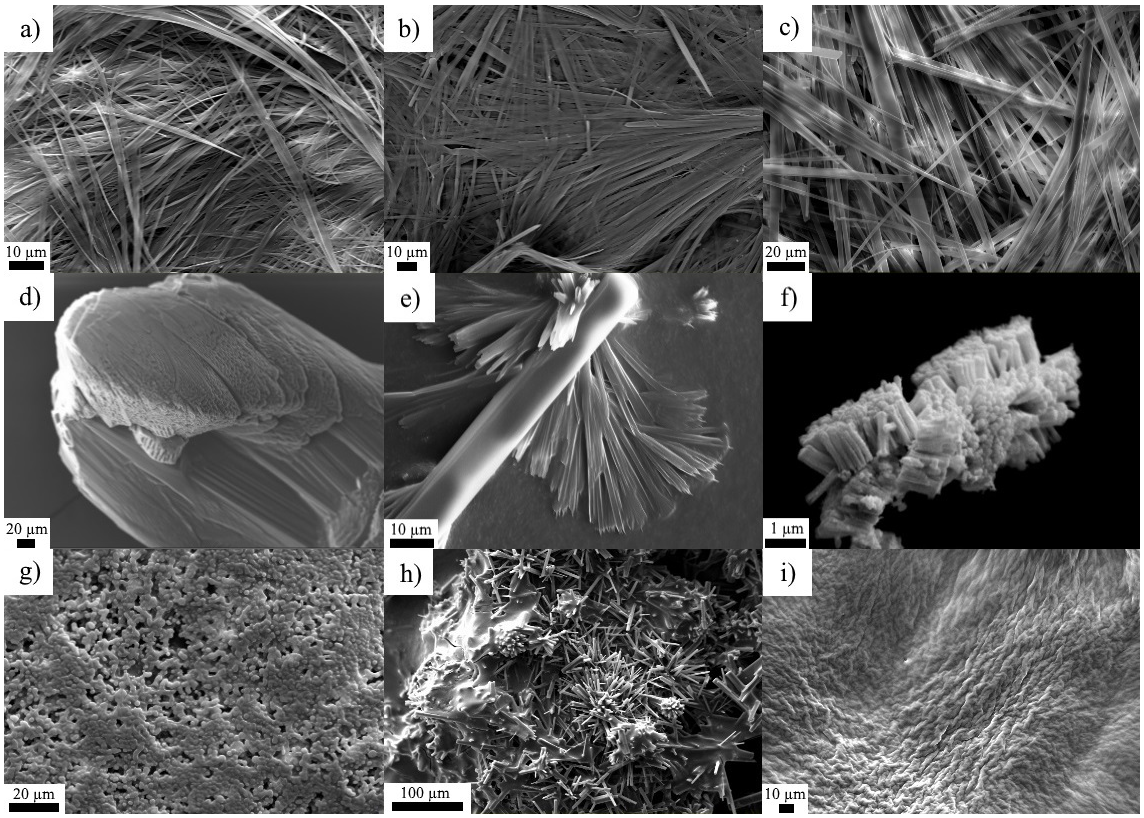


FIGURE 39 SEM images of compounds **124–132** in chlorobenzene as 2 % (w/v). a) gel of compound **124**, b) gel of compound **127**, c) gel of compound **130**, d) solution of compound **125**, e) solution of compound **128**, f) solution of compound **131**, g) gel of compound **126**, h) gel of compound **129**, and i) gel of compound **132**.

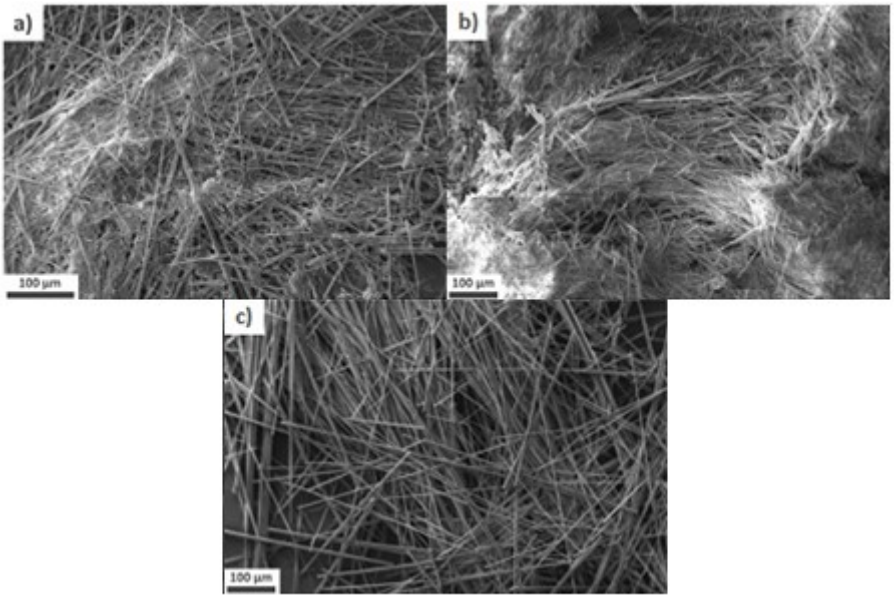


FIGURE 40 SEM images of 1 % (w/v) gels: compound **133** in benzene (a), compound **134** in benzene (b), and compound **136** in toluene (c).

The deoxycholyl derivatives did not act as gelators, excluding compound **134** containing hexyl side chain. Instead, they showed crystalline character in the SEM images as can be seen from Figure 39 d–f for compounds **125**, **128**, and **131**, respectively.

Compared to the gels of compounds **133** and **136**, compound **134** formed the shortest fibres (from 12 µm to 242 µm) in benzene (Figure 40 b) and medium sized fibres when regarding the width (from 732 nm to 4.7 µm). Interestingly, the gel formed by compound **134** was clearly more ductile. It seems that more elastic gel consists of relatively short fibres with varying length as well as medium-wide fibres with large variation in size, as is illustrated by compound **134** in benzene. However, larger fibres with more versatility in size and shape produce less elastic gels as exemplified by compound **133** in benzene.

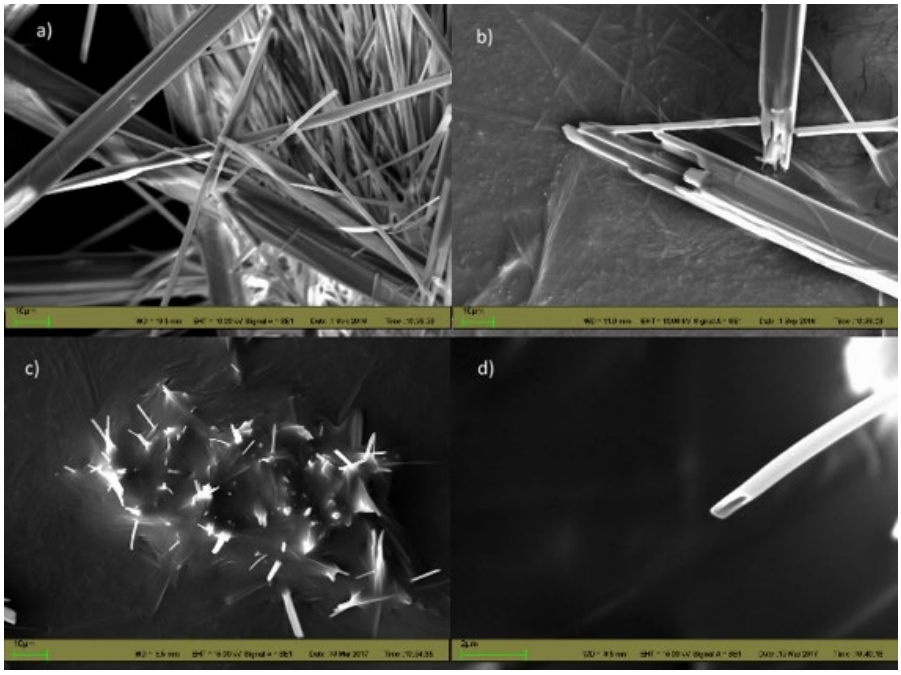


FIGURE 41 SEM images of the gels formed by compounds **124** (a and b) and **130** (c and d) in ethyl acetate as 1 % (w/v).

Cholic acid derivatives (compounds **126**, **129**, **132**, **135** and **138**) all formed spherical assemblies in chlorobenzene (Figure 39 g–i, compounds **126**, **129**, and **132**, respectively) or in benzene (Figure 42 a and b, compounds **135** and **138**, respectively) according to the SEM studies, some of which co-existed with crystalline material. Compound **126** possessed divergent spherical assemblies as 2 % (w/v) which were partly elided (Figure 39 g). Compound **129** assembled into ball-shapes of various sizes (Figure 39 h) which were consisted of rectangular crystals (width 21–67 μm and length 40–230 μm). The existence of crystals is possibly the reason why this compound with even numbered side chain (butyl) is such a poor gelator. On the contrary, compound **132** as 1 % (w/v) formed spherical assemblies as well which were similar to the ones formed by compound **126**, but significantly larger (120 μm in diameter in the 1 % gel) and with folded surface structure (Figure 43). As 2% compound **132** formed an extensively folded surface resembling double gauze fabric (Figure 39 i). Compound **135** formed smooth-surfaced spherical assemblies with various diameters (1.3–3.9 μm) as 1 % (w/v) as can be seen in Figure 42 a. Compound **138** self-assembled into more uniform-sized spheres of average diameter of 665 nm. The spherical assemblies were elided so that the gel network resembled a cookie dough (Figure 42 b). Regarding the mechanical strength of the gels, it is interesting that compound **135** with the mid-sized spherical assemblies with larger variation in the sizes, forms the strongest gel of all the 19 compounds studied.

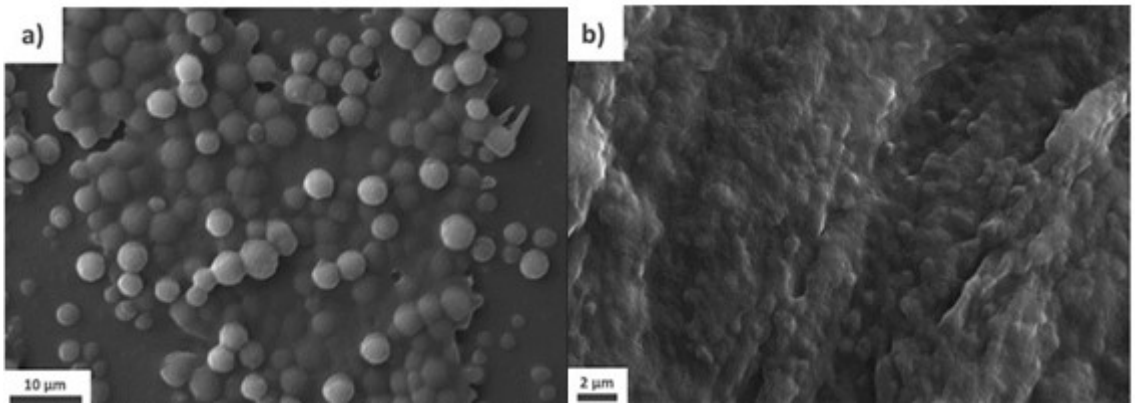


FIGURE 42 SEM images of the gels formed by compounds **135** (a) and **138** (b) in benzene as 1 % (w/v).

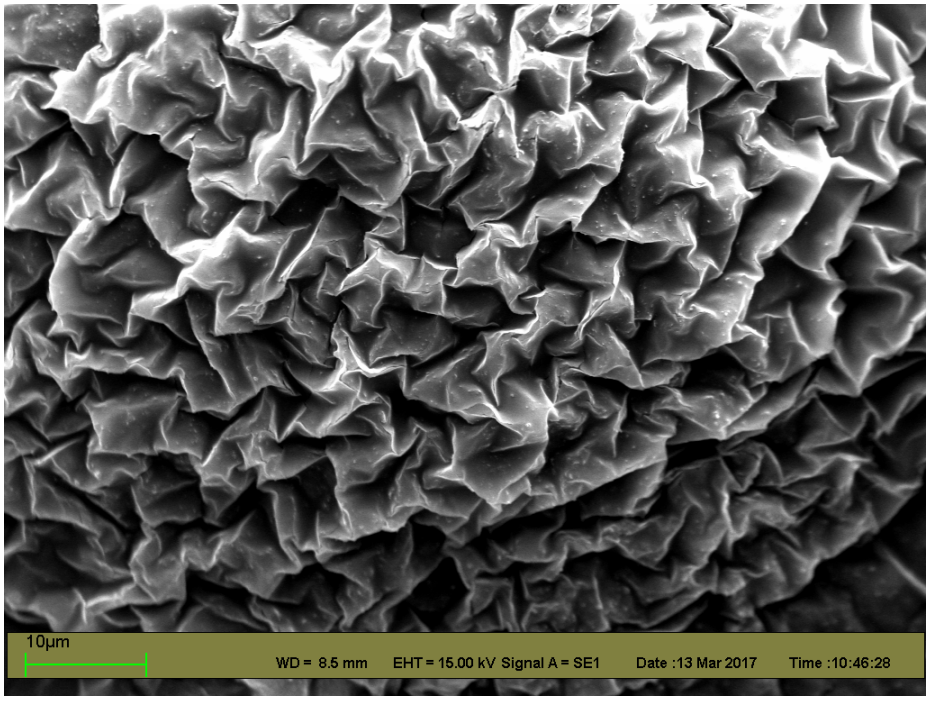


FIGURE 43 SEM image of the chlorobenzene gel formed by compound **132**.

2.5 Solid state studies of the compounds

2.5.1 Solid state NMR studies[II]

Solid state NMR spectroscopic studies of gel forming lithocholic acid derivatives (**124**, **127**, and **130**) and cholic acid derivatives (**126**, **129**, and **132**) were performed. All compounds were analyzed as re-crystallized from acetonitrile (compounds **124**, **126**, **127**, **129**, and **132**) or DMF (compound **130**) and as 2 % (w/v) chlorobenzene gels (except compound **130** as 4 % (w/v)). When comparing the ^{13}C CPMAS NMR spectra of the solid material and the gel, the spectra resembled each other in the case of lithocholic and cholic acid derivatives (Figure 44). The similarity of the solid-state spectra leads to the conclusion of the intermolecular interactions and chemical environments in the gel and solid state are alike.

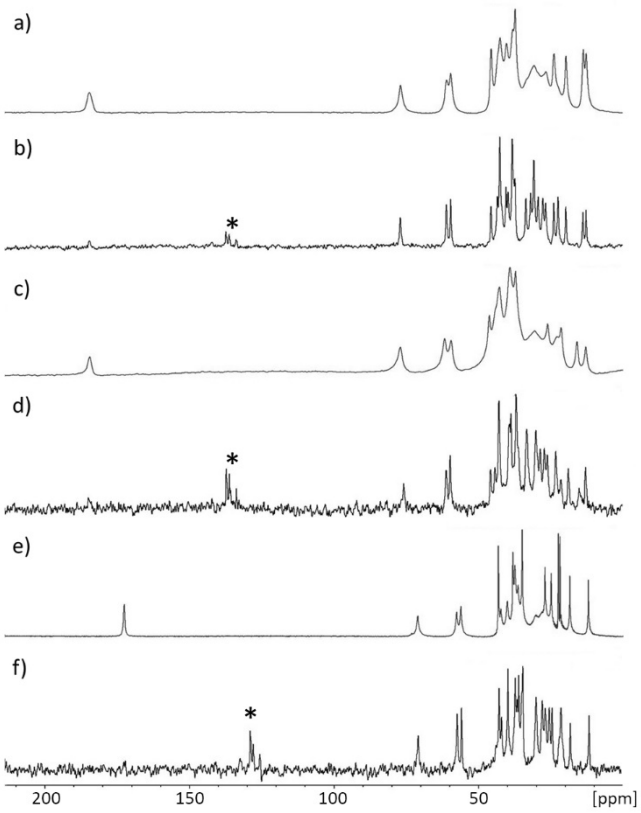


FIGURE 44 ^{13}C CPMAS NMR spectra of compounds **124**, **127**, and **130**. Compound **124** recrystallized from acetonitrile (a) and 2% (w/v) chlorobenzene gel of compound **124** (b). Compound **127** recrystallized from acetonitrile (c) and 2% (w/v) chlorobenzene gel of compound **127** (d). Compound **130** recrystallized from DMF (e), and 4% (w/v) chlorobenzene gel of compound **130** (f). Carbon signals from chlorobenzene in the gel samples are marked with an asterisk.

2.5.2 X-ray crystallographic studies[I–III]

Single crystal X-ray structures from majority of the compounds were achieved. Majority of the crystals were formed in acetonitrile and DMF.

Bile acid derivatives with ethyl side chain produced single crystals suitable for X-ray analysis from acetonitrile, excluding compound **123** with dehyrocholyl backbone. Compound **120** with lithocholyl backbone was observed to crystallize in monoclinic space group *P2*, with one molecule in the asymmetric unit. The alkylamide side chain (*tttt* conformation) and the 3α–OH group were oriented in the same direction (Figure 45 a). This conformation gave rise to infinite one-dimensional bilayers, which were stabilized by O–H···O and N–H···O hydrogen bonds (Figure 45 b). Compound **121**, containing deoxycholyl backbone, crystallized in triclinic spacegroup *P1* with four acetonitrile molecules in the asymmetric unit and three crystallographically independent molecules of compound **121**. The side chains in all three molecules of compound **121** were observed to be close to *ttgt* conformation. Intermolecular O–H···O and N–H···O hydrogen bonds were formed by 12α–OH groups, carbonyl oxygens and N–H groups. Additionally, hydrogen bonds were formed with acetonitrile molecules and peripheral 3α–OH groups, as depicted in Figure 45 c. Compound **121** was observed to crystallize into one-dimensional assemblies with columnar features, analogous to helices (Figure 45 d).

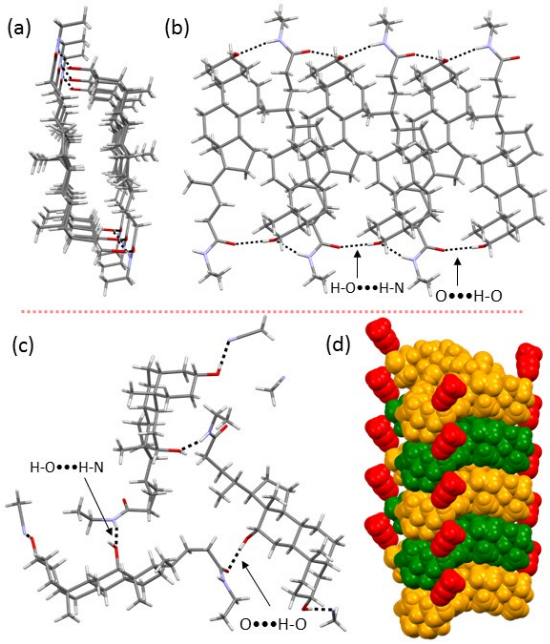


FIGURE 45 1-D Polymeric structure of compound **120** (a), and side-view to show 3α-hydroxyl group and *N*-ethylamide chain orientation in capped stick model (b). Top-view of 1-D hydrogen bonded trimeric structure of **121** in capped stick model (c). Side-view to stacking arrangement of trimers in **121** in CPK model (d). Representation: orange and green - compound **121**, and red - acetonitrile. Black broken lines represent hydrogen bonding.

The crystallization was more complex in the case of compound **122**, containing cholyl backbone with three hydroxyl groups. Compound **122** crystallized in monoclinic space groups *C2*. The asymmetric unit contained one acetonitrile molecule and two crystallographically independent molecules of compound **122** with divergent side chain conformations (*tgtg* and *tttt*) (Figure 46 a). As shown in Figure 46 b, the bilayer structure is formed by O–H⋯ O and N–H⋯ O hydrogen bonds from *tgtg* side chain conformation and the OH-groups on the concave amphiphilic side of the molecule. However, the side chain with *tttt* conformation forms hydrogen bonds from N–H group to the acetonitrile.

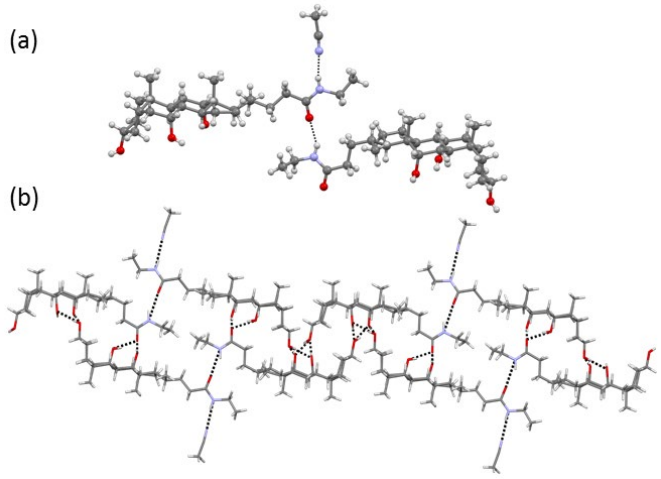


FIGURE 46 Asymmetric unit of compound **122** in ball and stick model (a), and section of crystal packing to show bilayer formation (capped stick model) (b). Hydrogen and solvent molecules are omitted for clarity. Black broken lines represent hydrogen bonding.

The single crystal structures of compounds **124–132** were observed to be monoclinic space group $P2_1$, thus creating a series of pseudo isomorphous and isostructural crystal structures. Crystals were obtained from acetonitrile, DMF or ethyl acetate. The difference was the lack of solvent molecules in the crystals, when compared to the ethyl side chain derivatives (compounds **120–122**), suggesting that the intermolecular interactions between the bile acid derivatives were preferable than interactions between the bile acid derivatives and the solvent molecules. The X-ray crystal structures of all compounds **124–132** are shown in Figure 47.

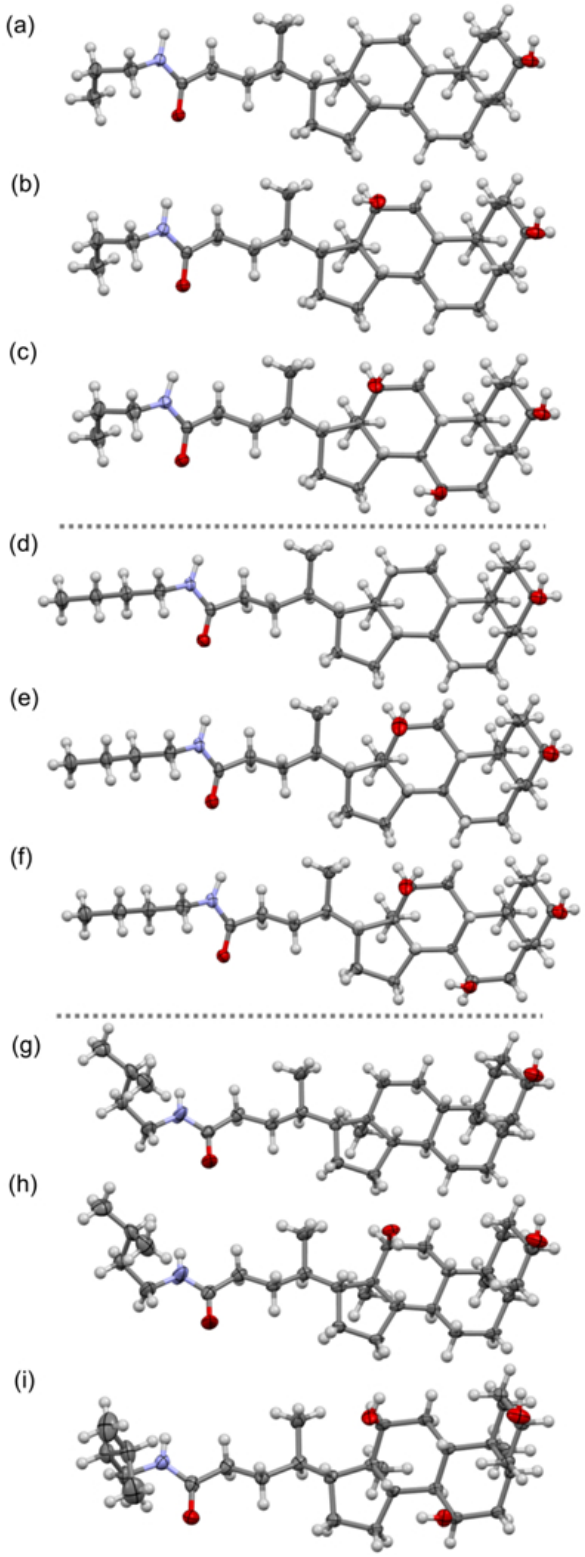


FIGURE 47 The X-ray crystal structures of compound **124** (a), compound **125** (b), compound **126** (c), compound **127** (d), compound **128** (e), compound **129** (f), compound **130** (g), compound **131** (h), compound **132** (i), with thermal ellipsoids at 50% probability level.

In the case of compounds **124–132**, typical one-dimensional bilayered structures were observed to be formed due to intermolecular O–H ··· O and N–H ··· O hydrogen bonds. Figure 48 depicts the bilayers formed by compounds **124**, **127**, and **130**. All amide and hydroxyl groups were fully utilized in these intermolecular interactions. Additionally, the one-dimensional bilayers were detected to form three-dimensional crystal lattice due to high percentage of H ··· H interactions by Hirshfeld surface analysis. In the Figure 48 the Hirshfeld surface analysis of compound **124** is presented.

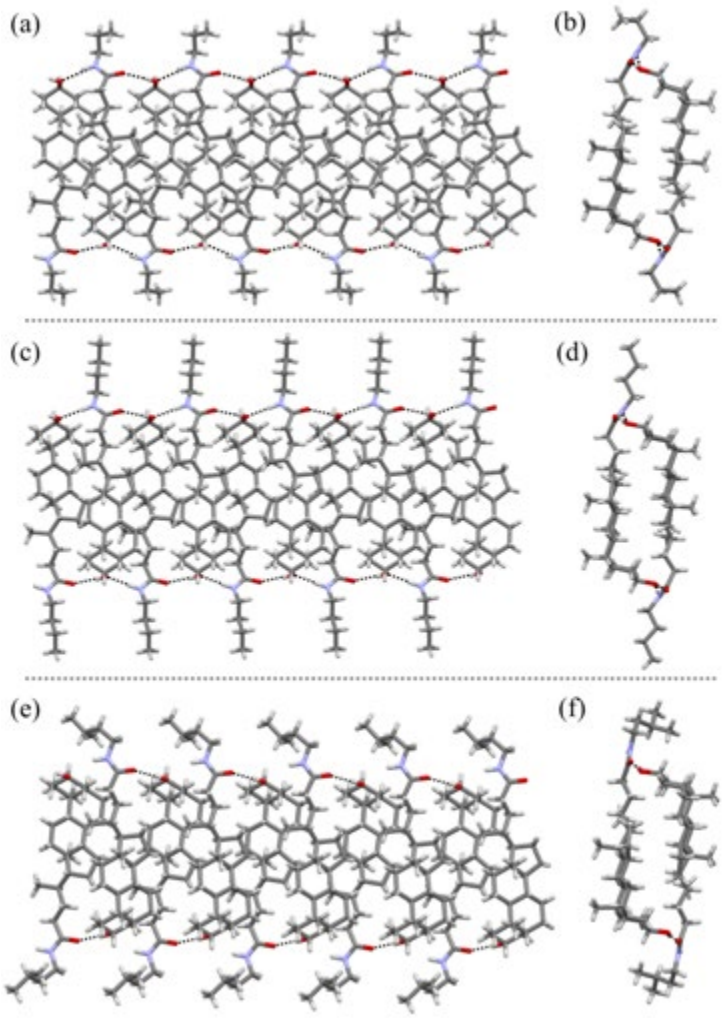


FIGURE 47 1-D hydrogen bond polymer viewed along *a*-axis of compounds **124** (a), **127** (c), and **130** (e). Tubular assemblies viewed along *b*-axis of compounds **124** (b), **127** (d), and **130** (f).

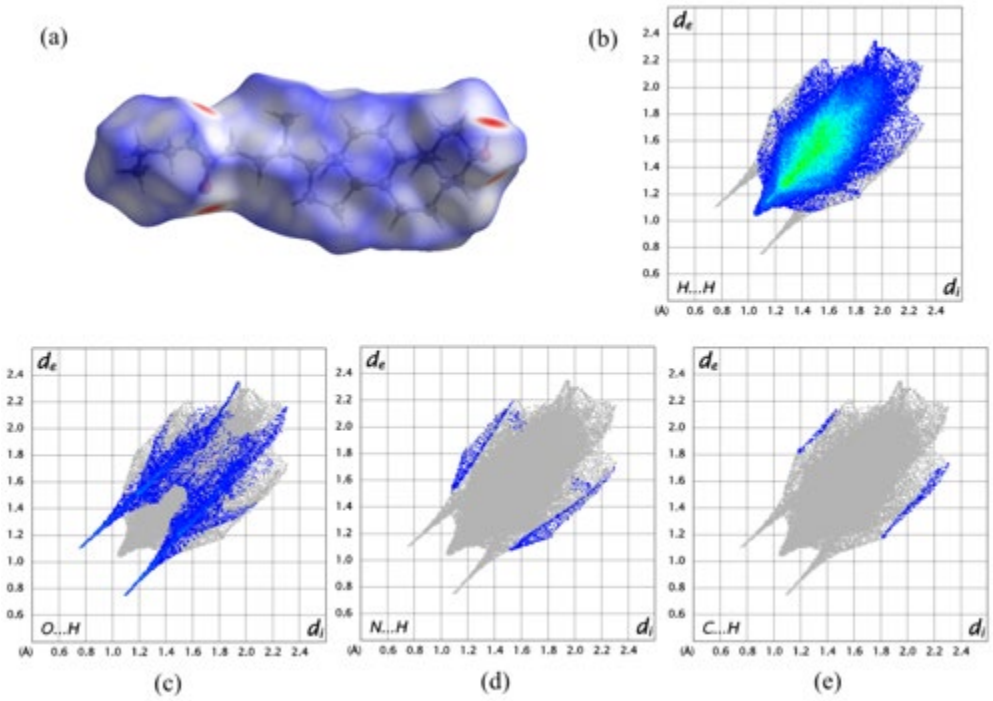


FIGURE 48 Hirshfeld surface for compound **124** mapped with d_{norm} (a). Fingerprint region of the compound **124** and intermolecular contacts to the Hirshfeld surface. H···H [90.3%] (b), O···H [8.9%] (c), N···H [0.6%] (d), and C···H [0.3%] (e). Colour scale is between -0.539 (red) and 1.362 (blue) au. The full fingerprint region is shown in gray scale. The d_i represents closest internal distance from a given point on the Hirshfeld surface, and d_e is the closest external contacts.

Single crystals suitable for X-ray diffraction analysis were achieved from compounds **133–137**. Crystallization occurred in acetonitrile or 1,4-dioxane. Compound **138** did not form any good crystals. In the Figure 49 solid state structures of compounds **133–137** are shown.

Compounds **133** and **135–137** crystallized in monoclinic space group $P2_1$, similarly as compounds **124–132**. Compound **134**, deoxycholic *N*-hexyl amide, was the exception as it produced crystals in high symmetry tetragonal space group *P43*. In all crystal structures obtained in this series of compounds, no solvent molecules were present in the crystal lattice. Interestingly, all other compounds produced crystals in which only one molecule is in the asymmetric unit except compound **135** with two crystallographically independent molecules.

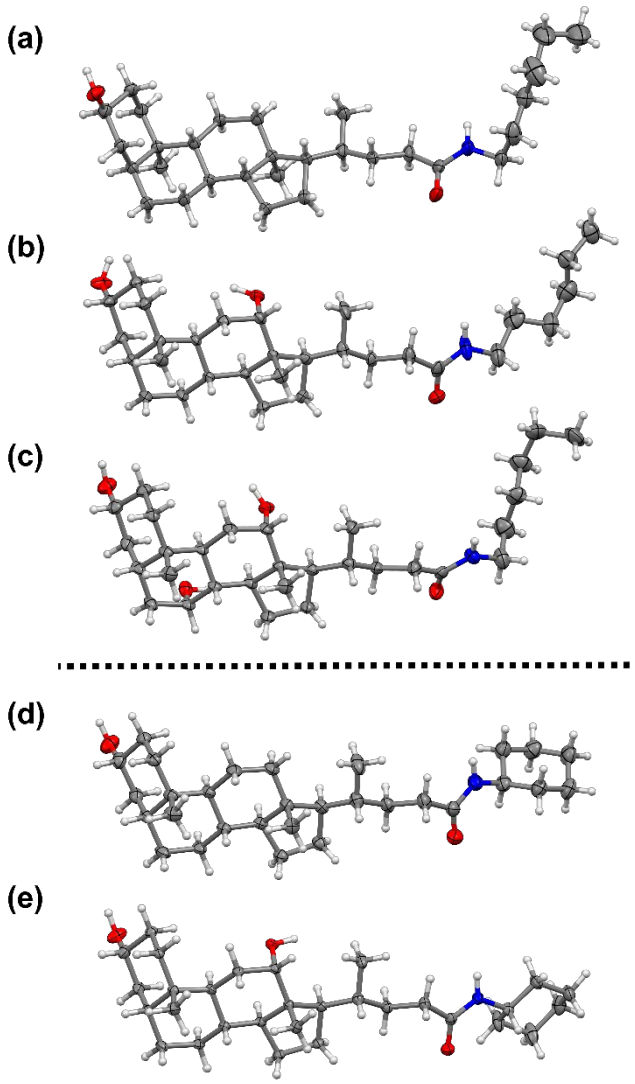


FIGURE 49 The X-ray structures of compounds **133–137**. Compound **133** (a), compound **134** (b), compound **135** (c), compound **136** (d), and compound **137** (e). Displacement ellipsoids are drawn at 50% probability level. Atom sites with minor occupancies have been omitted for clarity.

Intermolecular interactions between the bile acid derivatives were observed in the case of compounds **133**, **134**, **136**, and **137**, thus one-dimensional bilayered structures were observed. The bilayers were formed as a result of intermolecular O–H⋯ O and N–H⋯ O hydrogen bonds between the amide and hydroxyl groups, as is represented in Figure 50 (a and b) for compound **133**. Since compound **135** has two molecules in the asymmetric unit, the intermolecular interactions are divergent and produce two-dimensional hydrogen bond polymer. The hydrogen bond polymer forms by neighbouring one-dimensional bilayered and tubular assemblies interconnected by N–H⋯ O hydrogen bonds (Figure 50 c and d). In addition, Hirshfeld surface analyses of the crystal structures obtained showed formation of compact three-dimensional crystal

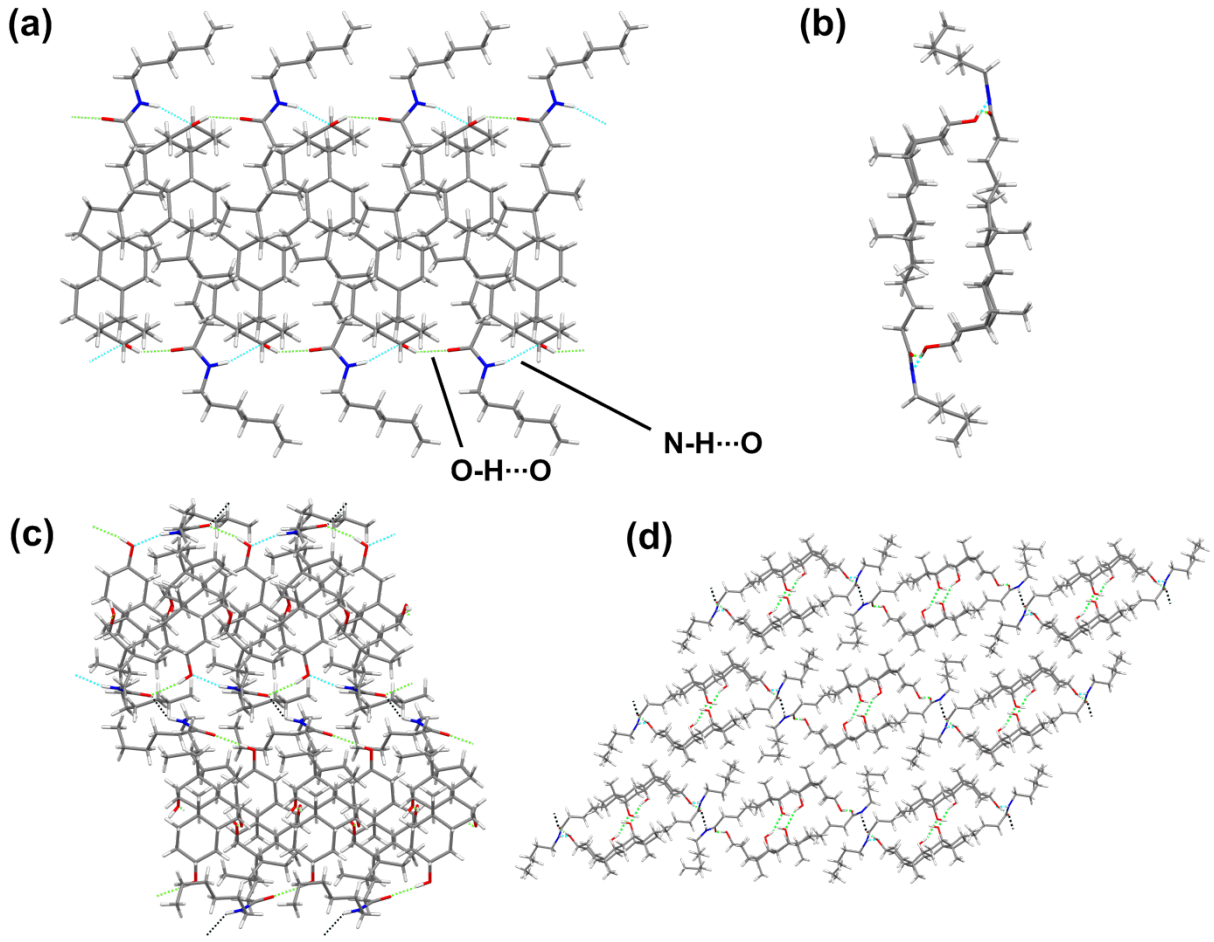


FIGURE 50 The 1D hydrogen bonded polymer viewed along a-axis (a) and tubular assembly viewed along b-axis of compound **133** (b). 2D hydrogen bond polymer viewed along a-axis (c) and b-axis of compound **135** (d) in capped stick model. Atom sites with minor occupancies have been omitted for clarity. Broken lines represent N-H···O (turquoise and black) and O-H···O (green) hydrogen bonding.

lattice from the bilayers by a high percentage of H···H contacts, similarly to compounds **124–132**.

In comparison to all other compounds discussed previously, compounds **133–138** are more flexible with respect of the side chains high degrees of conformational freedom. Also, as can be seen from Figure 51, the side chain is more flexible as the number of the hydroxyl groups in the steroidal backbone increases.

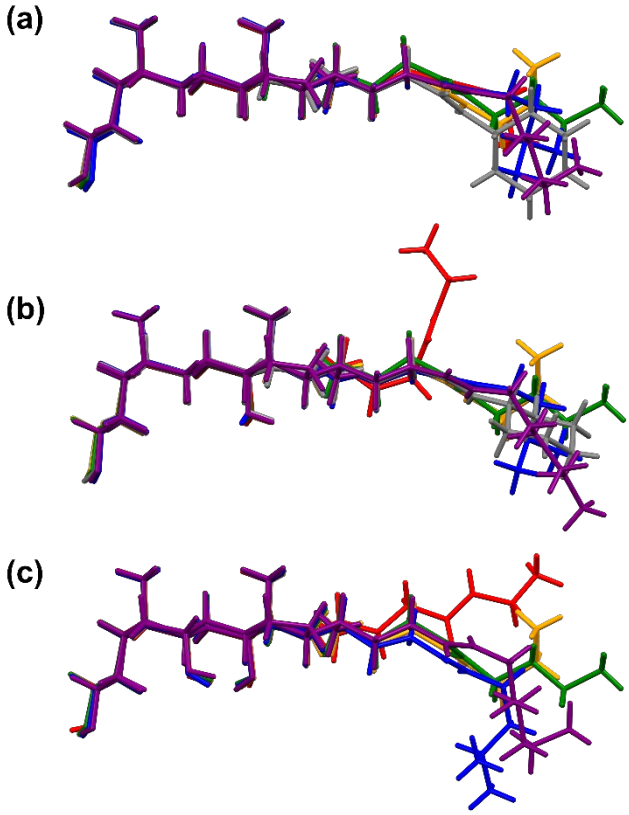


FIGURE 51 The overlay of the X-ray crystal structures of all lithocholyl (a), deoxycholyl (b), and cholyl (c) derivatives shown in capped stick model. Color codes: red (ethyl), gold (propyl), green (butyl), blue (*iso*-pentyl), grey (cyclohexyl), and purple (hexyl). Co-crystallized solvent molecules as well as atom sites with minor occupancies have been omitted for clarity.

2.6 Biological studies of ethyl amides[I]

Compounds **120–123** were studied with regard of their toxicity by using neutral red assay which is based on that non-damaged, live cell intake and store neutral red into their lysosomes. The dyes concentration can be detected by spectrophotometry. Half maximal inhibitory concentration (IC_{50}) was used to describe the compounds efficiency to inhibit biological function. Standard mouse cell line Balb/c 3T3 was utilized in the tests. Unfortunately, the lowest toxicity was implicated by compound **122** which did not form any gels. The only hydrogelator in the series, compound **120**, possessed second highest toxicity. The toxicity of the compounds **120–123** decreased as follows: **121** > **120** > **123** > **122**.

SUMMARY

Supramolecular gelation is a treasure box filled with immensely vast range of systems comprising of very divergent molecules and mixtures of them. This is the base from which stems numerous fascinating applications that may change our day-to-day lives in many aspects from electrical devises to health care. The structure of the potential gelator has an impact on the whether a gel is formed or not. When gelation is induced, the gelators' structure and the solvent effect on the properties of the gels. Furthermore, the self-assembly to a gel is quite unpredictable and many gelators are still discovered serendipitously today even though new techniques for gelator design have been developed.

Although the approach, in which a library of possible gelator molecules is prepared and then the self-assembly properties evaluated, is seen controversial in the study of why some molecules form gels and others do not, in this thesis work it is well-founded. With respect of one side chain there is three (or four in article I) divergent bile acid core molecules, that differ from each other by the number of hydroxyl groups. On the other hand, with respect of the same bile acid core, there is six divergent alkyl side chains. The bile acid core is very rigid; thus, it does not vary in shape when it is attached to different side chains, which means that the only thing effecting on the self-assembly properties is the side chain when observing compounds with same bile acid core. Additionally, in all of the molecules studied the side chain is attached to the bile acid core by an amide bond. This approach has led to intriguing conclusions of the gel formation and the properties of the gels of the compounds studied regarding different aspects.

The number of hydroxyl groups in the bile acid backbone, the even- and odd-numbered side chains and the shape of the side chain were observed to have an impact on the gelation properties of the compounds prepared. Hence, the family of compounds presented in this thesis provides some new information based on gelators structure and the gels formed. The formed gels were observed to be more stable with the lengthening of the side chain and the number of formed gel systems increased following the same pattern. However, cyclic side chain of compounds **136–138** produced less gels than corresponding hexyl side chain (compounds **133–135**). Even numbered side chains led to more good gels than weaker gels in the case of lithocholyl derivatives, whereas the opposite effect was observed when observing the cholyl derivatives.

Gelation was mostly observed in aromatic solvents. Kamlet-Taft analysis of the gelating solvents of compounds **133–138** revealed that the α-parameter is significant, as the value was zero for most of the solvents promoting gelation.

Morphology of the gels varied. Fibrous networks were seen in the case of lithocholyl derivatives. However, compound **120** formed fibres arranged to spherical assemblies. With other lithocholyl derivatives (compounds **124**, **127**, **130**, **133**, and **136**) fan-shaped fibrous assemblies were seen. The only deoxycholyl derivative capable to form gels, compound **134**, showed fibrous character also. Instead, cholyl derivatives (compounds **126**, **129**, **132**, **135**, and **138**) formed spherical assemblies exclusively. If the spherical assemblies consisted of crystals,

as in the case of compound **129**, the gel was observed to be mechanically weaker in addition to fewer gels formed. Interestingly, cholyl derivatives produced more stable gels when the variation of the size of spherical assemblies was large. For lithocholyl derivatives the opposite phenomenon was observed: greater variation of the fibre size resulted in weaker gels.

To conclude, multiple properties of the gelators and the formed gel network effect on the formation and stability of the gels. Lithocholyl and cholyl backbones promoted gelation predominantly, whereas deoxycholyl backbone did not. The length and shape were crucial when regarding the side chain and the structures forming the gel network.

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ORIGINAL PAPERS

I

BIOCOMPATIBLE HYDROGELATORS BASED ON BILE ACID ETHYL AMIDE

by

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Biocompatible hydrogelators based on bile acid ethyl amides

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ABSTRACT

Four novel bile acid ethyl amides were synthetized using a well-known method. All the four compounds were characterized by IR, SEM, and X-ray crystal analyses. In addition, the cytotoxicity of the compounds was tested. Two of the prepared compounds formed organogels. Lithocholic acid derivative **1** formed hydrogels as 1% and 2% (w/v) in four different aqueous solutions. This is very intriguing regarding possible uses in biomedicine.

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1. Introduction

Bile acids are a fascinating group of biologically important molecules belonging to the group of steroids [1,2]. Because of their relatively low cost, wide availability, and enantiomeric purity, bile acids are ideal building blocks for gelator molecules. The structure of the bile acids consists of a steroidal backbone and an aliphatic side chain [3]. The facially amphiphatic nature of bile acids arises from their concave hydrophilic face and convex hydrophobic face. Chemically different hydroxyl groups in the concave face and the varying amount of them, as well as the rigid steroidal backbone, are important with regard to bile acids' biological etc. properties. Because bile acids are endogenous compounds [4], they are perfect starting materials for biological and medical applications [3,5].

Bile acids and their derivatives have various applications especially in biomedicine. There are multiple examples in the field of cancer treatment [6–11]. One particularly interesting potential application involves mixing of sodium deoxycholate solutions to Au-nanoparticle (AuNP) solution to create multiple-branched AuNPs [8]. AuNPs formed have such a strong NIR absorption that they can be used to destroy tumor cells. Many examples include bile acid based compounds as drug carriers [12–17], and even gene [18,19] or RNA [20] carriers. Majority of potential applications of bile acids, or their derivatives, as drug carriers utilize bile acid transportation systems present in organisms/humans to achieve site-specific action of the drug carried. In addition, bile acid derivatives have shown potential as drug absorption modifiers [21,22].

Gels play increasingly important roles in modern life since they appear in myriad of applications ranging from optoelectronics [23,24] and biomedicine [23–25] to environmental clean-up [26,27]. Universally a gel is defined as a viscoelastic solid like material which consists of flexible cross-linked network and solvent. Gelator molecules form the cross-linked 3D network that attracts and captures solvent molecules. Gels are divided into different classes based on the solvent used. In hydrogels the solvent is pure water or water solution. If the solvent is organic, the gel is called an organogel, and dried gels are categorized as xerogels.

In the field of supramolecular gels the research focuses mainly on low molecular weight gelators (LMWG) [28]. Typically LMWGs form gel networks that resemble fibers. LMWGs consist of a rich variety of molecules to which bile acids and their derivatives enter into.

The first examples of bile acid salts forming hydrogels were reported in the early 20th century [29–31]. Despite of that there is a limited amount of bile acid salts and derivatives known to function as hydrogelators. Most of the bile acid based hydrogels reported have been discovered by Maitra and his co-workers. Maitra's research group has reported intriguing gel systems, including a tripodal cholamide supergelator [23]. This is, however, the first time bile acid alkyl amide derivatives are reported to gelate aqueous solutions.

Gels have potential use in biological applications only if they form in biocompatible solvents. Supramolecular hydrogels are

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considered to be biologically compatible, moreover they are formed in many cases by naturally occurring molecules which are most likely to be nontoxic [32]. Bile acid based hydrogels are considered to be fully biologically compatible when the gelator molecule itself is not toxic. The full biocompatibility of a hydrogel provides perhaps new potential applications regarding biomedicine, such as drug delivery, artificial tissue engineering, etc.

In relation to the previous work done by our research group [33–40], in this work we have focused on how the compounds we have prepared behave in aqueous media. As a continuation of the series of bile acid alkyl or functionalized alkyl amide/ester derivatives capable of acting as gelators we report four new bile acid ethyl amide based gelator molecules, which have shown potential in forming hydrogels.

2. Experimental

2.1. Materials

Lithocholic acid (⩾97%), deoxycholic acid (⩾99%), cholic acid (⩾97%), and dehydrocholic acid (⩾99.0%) were purchased from Sigma. Ethyl amide hydrochloride was purchased from Fluka. Triethyl amide, ethyl chloroformate, and other reagents used during the synthesis as well as solvents used in chromatography and gelation studies were of analytical grade. Ethyl chloroformate was distilled and 1,4-dioxane was dried over Na prior to use. All other chemicals were used without further purification. The synthetic route to **1**–**4** is presented in Scheme 1. The mixed anhydride method used has been previously reported by our research group [41].

2.2. General procedure for the synthesis of the bile acid ethyl amide conjugates ***1***–***4***

Reactions were performed under N_2 atmosphere. In a round-bottomed three-necked flask bile acid (5 mmol, 1 eq.) and 1,4-dioxane (42 mL) were cooled on an ice-water bath to +10 °C. To the cooled solution triethyl amide (6.7 mmol, 1.34 eq.) was added from a dropping funnel, followed by a dropwise addition of ethyl chloroformate (6.7 mmol, 1.34 eq.) in 1,4-dioxane (3 mL). The mixture was stirred at rt for 30 min. Meanwhile in another flask ethyl amide hydrochloride (6.7 mmol, 1.34 eq.) was suspended in DMF (10 mL). Suspension was cooled on ice-salt bath to 0 °C after which triethyl amide (7.4 mmol, 1.48 eq.) was added from a dropping funnel. The mixture was stirred at rt for 30 min. Ethyl amide in DMF was added dropwise to the bile acid anhydride and stirring of the reaction mixture continued at rt for 20 h. Volatiles were evaporated and the crude product was dissolved in $CHCl_3$ (100 mL). The crude product was washed with water (2 × 75 mL), 0.1 M HCl solution (2 × 75 mL), water (2 × 75 mL), and brine (2 × 75 mL). The organic layer was dried (Na_2SO_4), filtered, and the volatiles evaporated under reduced pressure. The crude products were purified by column chromatography (silica gel, DCM: MeOH 96:4 for **1**, 90:10 for **2**, *x*:*y* for **4**, and $CHCl_3$:MeOH 86:14 for **3**). All pure products were dried under vacuum.

2.2.1. Lithocholic acid ethyl amide ***1***

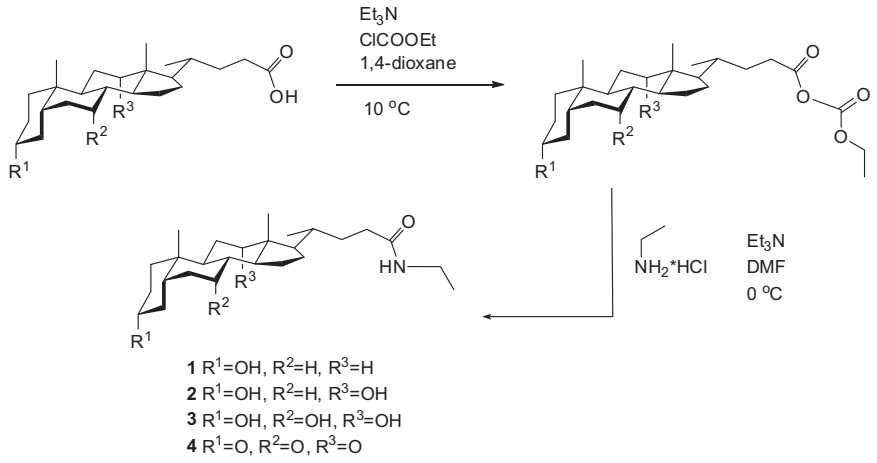
Yield 57%. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ 5.44 (t, 1H, NH), 3.62 (m, 1H, 3β-H), 3.28 (m, 2H, 25-CH_2), 2.21/2.04 (m, 2H, 23-CH_2), 1.14 (t, 3H, 26-CH_3), 0.91 (d, 3H, 21-CH_3), 0.91 (s, 3H, 19-CH_3), 0.64 (s, 3H, 18-CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz, ppm): δ 173.4 (C-24), 71.8 (C-3), 56.5 (C-14), 56.1 (C-17), 42.8 (C-13), 42.1 (C-5), 40.5 (C-9), 40.2 (C-12), 36.5 (C-4), 35.9 (C-8), 35.5 (C-20), 35.4 (C-1), 34.6 (C-10), 34.3 (C-25), 33.7 (C-23), 31.8 (C-22), 30.6 (C-2), 28.2 (C-16), 27.2 (C-6), 26.4 (C-4), 24.2 (C-15), 23.4 (C-21), 20.8 (C-7), 18.4 (C-19), 14.9 (C-26), 12.0 (C-18). MS: *m*/*z* 426.7 $[M+Na]^+$, 830.5 $[2M+Na]^+$.

2.2.2. Deoxycholic acid ethyl amide ***2***

Yield 75%. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ 5.50 (t, 1H, NH), 3.97 (m, 1H, 12β-H), 3.61 (m, 1H, 3β-H), 3.28 (m, 2H, 25-CH_2), 2.23/2.06 (m, 2H, 23-CH_2), 0.97 (d, 3H, 21-CH_3), 0.90 (s, 3H, 19-CH_3), 0.65 (s, 3H, 18-CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz, ppm): δ 173.4 (C-24), 73.2 (C-12), 71.8 (C-3), 48.3 (C-14), 47.2 (C-17), 46.5 (C-13), 42.1 (C-5), 36.4 (C-4), 36.0 (C-8), 35.2 (C-1, C-20), 34.4 (C-25), 34.1 (C-10), 33.7 (C-9), 33.5 (C-23), 31.7 (C-22), 30.5 (C-2), 28.7 (C-11), 27.5 (C-16), 27.1 (C-6), 26.1 (C-7), 23.7 (C-15), 23.2 (C-19), 17.5 (C-21), 14.9 (C-26), 12.8 (C-18). MS: *m*/*z* 442.7 $[M+Na]^+$, 862.5 $[2M+Na]^+$.

2.2.3. Cholic acid ethyl amide ***3***

Yield 53%. ^{1}H NMR ($CDCl_3$, 500 MHz, ppm): δ 5.76 (t, 1H, NH), 3.96 (m, 1H, 12β-H), 3.84 (m, 1H, 7β-H), 3.44 (m, 1H, 3β-H), 3.27 (m, 2H, 25-CH_2), 2.21/2.09 (m, 2H, 23-CH_2), 0.99 (d, 3H, 21-CH_3), 0.88 (s, 3H, 19-CH_3), 0.68 (s, 3H, 18-CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz, ppm): δ 173.6 (C-24), 73.1 (C-12), 72.0 (C-3), 68.4



Scheme 1. The synthetic route leading to compounds **1**–**4**.

(C-7), 46.8 (C-17), 46.5 (C-13), 41.9 (C-14), 41.5 (C-5), 39.7 (C-4), 39.6 (C-8), 35.3 (C-1, C-20), 34.7 (C-6, C-10), 34.4 (C-23), 33.3 (C-25), 31.7 (C-22), 30.6 (C-2), 28.3 (C-11), 27.5 (C-16), 26.6 (C-9), 23.2 (C-15), 22.5 (C-19), 17.5 (C-21), 14.9 (C-26), 12.6 (C-18). MS: *m*/*z* 458.8 $[M+Na]^+$, 474.8 $[M+K]^+$, 894.6 $[2M+Na]^+$.

2.2.4. Dehydrocholic acid ethyl amide ***4***

Yield 34%. ^1H NMR ($CDCl_3$, 500 MHz, ppm): δ 5.50 (t, 1H, NH), 3.27 (m, 2H, 25-CH_2), 2.88 (m, 1H, 8-CH), 1.39 (t, 3H, 26-CH_3), 1.12 (d, 3H, 21-CH_3), 1.06 (s, 3H, 19-CH_3), 0.84 (s, 3H, 18-CH_3). ^{13}C NMR ($CDCl_3$, 126 MHz, ppm): δ 211.9 (C-12), 208.9 (C-3), 208.6 (C-7), 173.1 (C-24), 56.9 (C-13), 51.8 (C-14), 48.9 (C-8), 46.8 (C-5), 45.6 (C-17), 45.5 (C-9), 44.9 (C-6), 42.8 (C-4), 38.6 (C-11), 36.4 (C-2), 35.9 (C-10), 35.5 (C-20), 35.2 (C-1), 34.3 (C-25), 33.6 (C-23), 31.2 (C-22), 27.6 (C-15/C-16), 25.1 (C-15/C-16), 21.9 (C-26), 18.7 (C-18), 14.9 (C-21), 11.8 (C-19). MS: *m*/*z* 452.7 $[M+Na]^+$, 484.8 $[M+MeOH+Na]^+$.

2.3. NMR spectroscopy

^1H, ^{13}C, and 2D ^1H, ^{13}C HMQC and HMBC spectra used for characterization of the compounds **1**–**4** were recorded with a Bruker Avance DRX 500 MHz spectrometer. The spectrometer was equipped with 5 mm diameter broad band inverse detection probehead operating at 500.17 MHz in ^1H and 125.77 MHz in ^{13}C experiments, respectively. The ^1H NMR chemical shifts are referenced to the signal of residual $CHCl_3$ (7.26 ppm from internal TMS). The ^{13}C NMR chemical shifts are referenced to the center peak of the solvent $CDCl_3$ (77.0 ppm from internal TMS). A composite pulse decoupling, Waltz-16, has been used to remove proton couplings from ^{13}C NMR spectra.

2.4. Mass spectrometry

Compounds **1**–**4** were studied by mass spectrometry. Measurements were performed by using Micromass LCT time of flight (TOF) mass spectrometer with electrospray ionization (ESI). Measurements were conducted using positive ion mode. MassLynx NT software system was used to control the spectrometer, and to acquire and process the data. Flow rate for the sample solutions was 10 μl/min. Sample cone and extraction cone potentials were 40 V and 6 V, respectively. The capillary cone potential varied between 3600 V and 4000 V, RF lens potential was 250 V in all measurements. The desolvation temperature was set to 120 °C and the source temperature to 80 °C.

Stock solutions of compounds **1**–**4** were 1 mM in acetone. Measurement solutions were prepared from stock solutions by diluting them to 10 μM or 20 μM in methanol.

2.5. IR-spectroscopy

Compounds **1**–**4** were studied by IR-spectroscopy. Measurements were conducted using Bruker Tensor 27 Fourier transform IR-spectrometer equipped with GladiATR™ accessory in the range of 400–4000 cm^{-1}. Compounds **1**–**4** were measured in solid state. Hydrogels formed by compound **1** in 2% (w/v) in 50:50 H_2O:2-propanol, and 10:90 H_2O:acetone and H_2O:acetonitrile were also studied. For the measurements of the hydrogels of compound **1**, the solution system in question was measured and the spectrum obtained was subtracted from the spectrum of the gel to eliminate the background signal.

2.6. Gelation studies

Self-assembly properties of compounds **1**–**4** were studied by weighing 5 mg to obtain 1% (w/v) systems or 10 mg to obtain 2% (w/v) systems of a particular compound in a test tube and adding 500 μl of solvent or solvent mixture in question. The mixture was subjected to ultrasound for ca. 1 min with the cap on and heated with a heat gun until the compound was dissolved or the boiling point of the solvent reached without the cap. The solution or suspension was cooled to rt and the observations with regard gelation were made within 1–2 h after cooling had commenced. Formed solid mass was defined as a gel if there was no solvent flow when test tube was inverted. Possible crystallization and precipitation was observed within days or weeks. Test tubes were stored at rt during the observation time.

2.7. SEM

Samples for SEM were prepared by a traditional method. The hot solution containing the gelator in question in an appropriate solvent system was pipetted to the sample stub, and the gel allowed to form while cooling to room temperature. In addition to the traditional method, samples were prepared also directly from previously formed gels. A small amount of a gel in question was scooped with a spatula and the sample stub was thinly brushed with the gel. The gel was allowed to dry on the sample stub in both methods of sample preparation. After drying, all samples were thinly plated with gold with JOEL Fine Coat Ion Sputter JFC-1100. Micrographs were taken with Bruker Quantax400 EDS scanning electron microscope equipped with a digital camera.

2.8. Toxicity evaluation

2.8.1. Cell culture

Mouse fibroblasts Balb/c 3T3 (No. 86110401) was obtained from European Collection of Cell Cultures (UK). Cell line stored in a cryovial were taken out of the liquid nitrogen and thawed in water (25 °C; 1 min). Using a pipette, cells were transferred into a 25 cm^2 tissue culture flask containing 10 ml of pre-heated culture medium (DMEM, penicillin 100 U/ml, L-glutamine 2 mmol/l, streptomycin 100 mg/l, fetal calf serum 5%, new born calf serum 5%) and incubated in humidified atmosphere with 5% (v/v) CO_2 at 37 °C. The medium was changed every 24–48 h. Cells were cultivated to approach confluence and were passaged. The medium was replaced and cells were washed with PBS (5 ml). Solution was removed and 0.25% trypsin–EDTA was added (0.5 ml, 2–3 min, 37 °C). Then 5 ml of culture medium was applied, cells were centrifuged (10 min; 1300 rpm; RT) and the supernatant was removed. The cell pellet was resuspended in 20 ml of culture medium and suspension was transferred to the 75 cm^2 cultivating flask. During cultivating in a 75 cm^2 tissue culture flask the procedure was the same, only 10 ml of PBS, 1 ml of trypsin–EDTA and 10 ml of culture medium were used.

2.8.2. Sample preparation

Stock solutions of samples **1** and **2** (0.039–5 mg/ml) and **3** and **4** (0.156–20 mg/ml) were prepared in DMSO. The final concentration of DMSO in serum-free medium was 0.5% (v/v). The DMSO was added to the compounds, thoroughly mixed (2 min), and sonicated (10 min). Control cells were incubated with a corresponding volume of DMSO.

2.8.3. Procedure

The concentrations of the cells were determined using colored with trypan blue. The cells were seeded in 96-well plates at a density of 0.8×10^5 cells/ml (200 μl/well) in culture medium and incubated for 24 h in a humidified atmosphere (37 °C, 5% CO_2). Then the culture medium was change to the serum-free one containing test compounds in concentration ranges of 0.195–25 μg/ml for **1** and **2**, and 0.781–100 μg/ml for **3** and **4**, and incubated for 24 h

(37 °C, 5% CO_2). After incubation period the cell damage was evaluated as the incorporation of neutral red into the lysosomes of living cells (Neutral red assay). The test was performed in 3 independent experiments.

2.8.4. Neutral red assay

The neutral red (NR) assay is grounded on the fact that live (non-damaged) cells intake and store NR into their lysosomes. The concentration of the incorporated dye is determined spectrophotometrically at 540 nm after extraction of retained NR into acidic methanolic solution [42].

After the incubation period the cells were first washed with PBS, and subsequently NR solution (0.03%, w/v in PBS) was applied to the cells for 3 h (37 °C, 5% CO_2). Then the cells were washed with a washing solution (formaldehyde (0.125%; v/v), $CaCl_2$ (0.25%, w/v)), and the retained NR was dissolved in extraction solution (methanol (50%; v/v), acetic acid (1%; v/v)). The absorbance was measured with a microplate reader (Sunrise Remote, Tecan, Austria).

2.9. X-ray crystallography

Single crystal X-ray data for **1**, **2**, and **3** were collected at either 120 or 123 K using Agilent Super-Nova dual source wavelength diffractometer with an Atlas CCD detector using multilayer optics monochromatized CuK_α (λ = 1.54184 Å) radiation. The data collection and reduction for **1**, **2**, and **3** was performed using the program *CrysAlisPro* [43]. Gaussian face index absorption correction method43] was used for **1**, **2**, and **3**. All the structures were solved with direct methods (*SHELXS*) [44] and refined by full-matrix least squares on F^2 using the *OLEX2* [45], which utilizes the *SHELXL*-2013 module [44]. No attempt was made to locate the hydrogens for solvent molecules and heteroatoms, and all the hydrogen atoms were added using ADD H command in *OLEX2*. Attempts to solve the disorder of N-ethyl chain in compound **3** still engenders B-alerts and requires the usage of constraints (EADP) and restraints (ISOR), which ultimately affects the R_1-value significantly. As a result, considering the conformational importance of side chain during our discussion, no attempt was made to solve the disorder.

3. Results and discussion

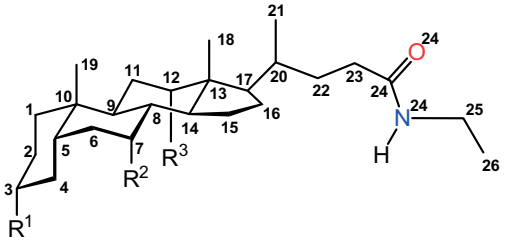
3.1. Synthesis

A well-established method, employed several times by our research group [41,46], was used to synthesize compounds **1**–**4**. Syntheses were relatively fast compared to syntheses that last several days [46]. Purification of compounds **3** and **4** was challenging, probably due to the three hydrophilic functional groups of the steroidal backbone which cause stronger interactions with silica gel when using column chromatography. Yields varied between 34% and 75%.

3.2. Gelation studies

Gelation properties of compounds **1**–**4** (Fig. 1) were tested in 28 solvents at 1% (w/v) and in various aqueous solutions at 1% and 2% (w/v) concentration. The results obtained are summarized in Tables 1 and 2, respectively.

A total of 19 gel systems were obtained, most of which in aromatic solvents. Five of the obtained gel systems were partial gels. Aromatic solvents included benzene, chlorobenzene, *tert*-butylbenzene, toluene, *p*- and *o*-xylene, and anisole. Compound **1** formed gels in acetone and acetonitrile in addition to aromatic solvents. Partial gels of compound **1** formed in *m*-xylene, mesitylene, and ethylacetate. Compound **4** formed 2% (w/v) gel in ethanol and



1 R^1=OH, R^2=H, R^3=H
2 R^1=OH, R^2=H, R^3=OH
3 R^1=OH, R^2=OH, R^3=OH
4 R^1=O, R^2=O, R^3=O

Fig. 1. Chemical structures of compounds **1**–**4**.

Table 1
Gelation test results for compounds **1**–**4** at 1% (w/v) concentration.

Solvent	1	2	3	4
$CHCl_3$	S+	S+, S	S+	S+
DCM	PS+, S	S+, S	PS+, S, P	S+
CCl_4	PS+, PS	PS+, PS	PS+, PS	PS+, PS
Benzene	PS+, PS, **G**	PS, S	PS+, PS	PS+, PS
Chlorobenzene	PS+, S, **G**	PS+, S	PS+, S	PS+, S
tert-Butylbenzene	PS+, S	PS+, PS	PS+, S	PS+, S, P
Toluene	PS+, PS, **G**	PS+, S, PS	PS+, S	PS+, S
p-Xylene	PS+, S, **G**	PS+, S, PS	PS+, S	PS+, PS
m-Xylene	PS+, S, PG	PS+, S, PS	PS+, S	PS+, PS
o-Xylene	PS+, S, **G**	PS+, S, PS	PS+, S	PS+, S
Cumene	PS+, S	PS	PS+, S	PS+, PS
Mesitylene	PS+, S, PG	PS+, S, PS	PS+, S	PS+, S
Anisole	PS+, S, **G**[a]	PS+, S, PS	PS+, S	PS+, S
Cyclohexene	S+	PS+, S	S+	PS+, S, P
Ethylacetate	PS+, PS, PG	PS+, S	PS+, S, P	PS+, S
Hexane	PS+, PS	PS+, PS, P	PS+, PS	PS+, PS, P
Acetone	PS+, PS, **G**	PS+, S	PS+, S	PS+, S
ACN	PS+, PS, **G**	–	PS+, S	S+
DMF	PS+, S	S+, S	S+	S+
Acetic acid	S+	S+, S	S+	S+
THF	S+	S+, S	S+	PS+, S
MeOH	S+	S+, S	S+	PS+, S, P
EtOH	S+	S+, S	PS+, S	PS+, PS
2-Propanol	S+	S+, S	S+	PS+, PS
1-Butanol	S+	S+, S	S+	PS+, S, P
1-Octanol	S+	PS+, S	S+	PS+, S, P
Cyclohexanol	PS+, S	PS+, S	PS+, S	PS+, S
2 M HCl	PS+, PS, P	PS+, PS	PS+, PS	PS+, PS, P
Deionized H_2O	PS+, PS, P	PS+, PS, P	PS+, PS, P	S+
Pyridine	S+	S+, S	S+	PS+, PS

S+ = soluble without heating, S = soluble with heating, PS = partially soluble with heating, I = insoluble, G = gel with appr. 0–5% free solvent, G- = gel with appr. 5–20% free solvent, G^a = gel formed after one day, PG = gel with appr. 50% free solvent, P = precipitates on cooling.

a 2% (w/v) weak gel in 2-propanol. Lithocholic acid derivative **1** formed the majority of the gel systems, a total of 16 gel systems, which is common for bile acid derivatives. For example, most of the gel systems from the bile acid cysteamine and alkylamide derivatives reported previously by our research group tended to be formed by lithocholic acid derivatives [37,38]. Some of the gel systems in previous studies were obtained with dehydrocholic acid derivatives, as well [35].

In the present study we found that lithocholic acid derivative **1** had interesting properties in water solutions. The solution systems for 1% (w/v) hydrogelation tests were selected based on the gel systems obtained in organogelation tests. Furthermore, the solution systems for 2% (w/v) hydrogelation tests were chosen based on results from previously conducted 1% (w/v) hydrogelation tests.

Table 2
Gelation test results for compounds **1**–**4** at 1% (w/v) concentration in aqueous solutions.

	1	**2**	**3**	**4**
50:50				
H_2O:MeOH	PS+, PS, P	PS+, S, P	PS+, PS	PS+, PS
H_2O:EtOH	PS, P	S	S	S
H_2O:acetone	PS, P	S	S	S
H_2O:ACN	PS, P	S, P	S	S, P
H_2O:2-propanol	PS, P, gel-like	S	S	S+
60:40				
H_2O:MeOH	PS+, PS, P	PS+, PS, P	PS+, S, PS	PS+, PS
H_2O:EtOH	PS, P	S, P	S	PS, P
70:30				
H_2O:MeOH	PS+, PS, **G**	PS+, PS, suspension	PS+, PS, S	PS+, PS
H_2O:EtOH	PS, P	PS, P	S+, P	PS, P
80:20				
H_2O:EtOH	PS, P	S+, P	PS, P	PS, P
10:90				
H_2O:EtOH	S+	S+	S+	S+
H_2O:acetone	S, **G**–	S+	S	S
H_2O:ACN	S+, P, gel-like	S	S	S
H_2O:2-propanol	S+	S+	S	S+
20:80				
H_2O:EtOH	S	S+	S+	S+
30:70				
H_2O:EtOH	S+	S	S	S+
H_2O:acetone	PS, P	S	S	S
H_2O:ACN	PS, P	S	S	S+, P
H_2O:2-propanol	S	S	S	S+

S+ = soluble without heating, S = soluble with heating, PS = partially soluble with heating, I = insoluble, G = gel with appr. 0–5% free solvent, G- = gel with appr. 5–20% free solvent, G[a] = gel formed after one day, P = precipitates on cooling.

Table 3
Selected IR bands for compounds **1**–**4** and hydrogels of compound **1**.

Sample	Amide I (cm^{-1})	Amide II (cm^{-1})	Amide B (cm^{-1})	νNH/OH (cm^{-1})
1	1655	1546	2932	3325, 3432
2	1624	1530	2926	3369
3	1612	1557	2927	3281, 3419, 3511
4	1701	1543	2970	3287, 3391
2% **1** in 50:50 H_2O:2-propanol	1655	1545	2932	3322, 3431
2% **1** in 10:90 H_2O:acet-one	1655	1545	2932	3326, 3435
2% **1** in 10:90 H_2O:acet-onitrile	1655	1545	2932	3324, 3434

Compound **1** generated five hydrogel systems, of which three were strong gels. The 1% (w/v) weak gel by compound **1** formed in 10:90 H_2O:acetone. Two of the three strong gels by compound **1** formed as 2% (w/v) in 50:50 H_2O:2-propanol and 10:90 H_2O:acetonitrile. One strong gel formed as 1% (w/v) in 70:30 H_2O:MeOH. Compound **1** also formed a partial gel as 2% (w/v) in 10:90 H_2O:acetone. In addition to actual gel systems, compound **1** formed gel-like systems as 1% (w/v) in 50:50 H_2O:2-propanol and 10:90 H_2O:acetonitrile. Compound **2** was found to create partial gel as 2% (w/v) in 70:30 H_2O:MeOH. Interestingly, the three proper hydrogels disintegrated instantly when disturbed with a spatula making them thixotropic by nature. The same phenomenon was observed for compound **1** 1% (w/v) gel in acetonitrile when preparing the SEM samples.

3.3. IR

The IR spectra of the solid compounds **1**–**4** were very similar with each other showing just a little variation in the amide bond's carbonyl stretching mode (Table 3). The solid compound **1** and the 2% hydrogels (50:50 H_2O:2-propanol, 10:90 H_2O:acetone and H_2O:acetonitrile) of compound **1** were rather uniform when compared to each other. In the spectrum of 2% (w/v) hydrogel of compound **1** in 10:90 H_2O:acetone, however, a broad band at 3300–3500 cm^{-1} was observed (Fig. 2), probably due to hydrogen bonding of the gelator molecules. Since, based on X-ray crystallography, hydrogen bonding is important in stabilizing the solid state structures, and since the difference to the spectrum of solid compound **1** is so small, hydrogen bonding in not necessarily the only driving force in the gel network formation.

3.4. Microscopy

The hydrogels formed by compound **1** were studied with scanning electron microscope (SEM). When comparing the SEM images of gels formed by compound **1** in aqueous solutions, it was found that the self-assembly patterns diverge from each other depending on the method of sample preparation. In the case of samples prepared from hot solutions, gel fibers resembled stalks of grass. However, gelator molecules appeared to form spherical assemblies consisting of fiber bundles resembling old fashioned dusters in samples prepared from gels directly. The assemblies of these bundles were a spitting image of dandelions fluffy seeds. This was most clearly seen in images of the 2% (w/v) gel of compound **1** in 50:50 H_2O:2-propanol (Fig. 3).

In all the gels studied the fiber sizes were similar. Straight fibers in samples prepared from hot solutions varied in width between 1 and 15 μm and in length between 20 and 240 μm. In samples prepared by scooping a portion of the gel on the sample stub single fibers were similar in width compared to stalk like fibers. However,

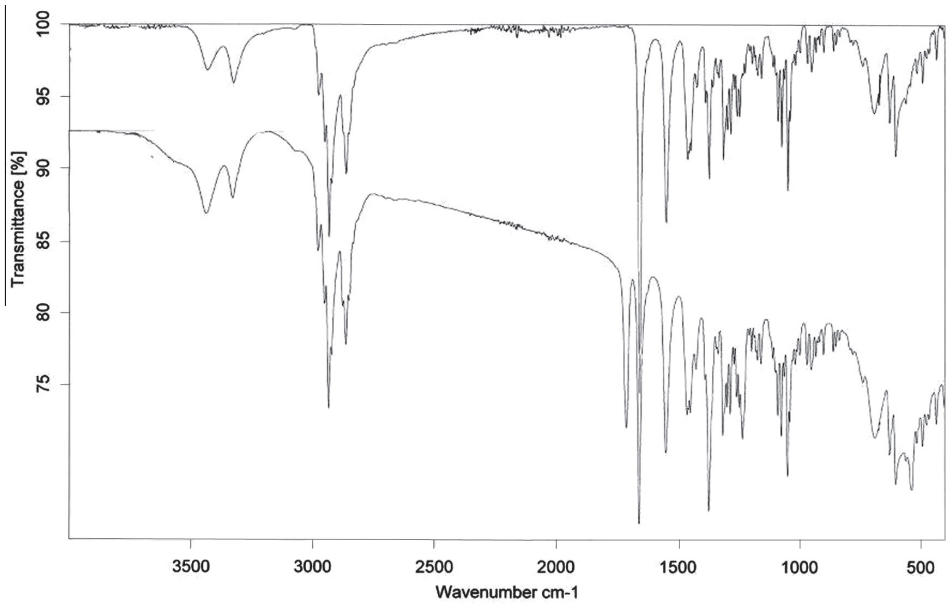


Fig. 2. IR spectra of compound **1** (above) and 2% (w/v) gel of compound **1** in 10:90 H2O:acetone (below).

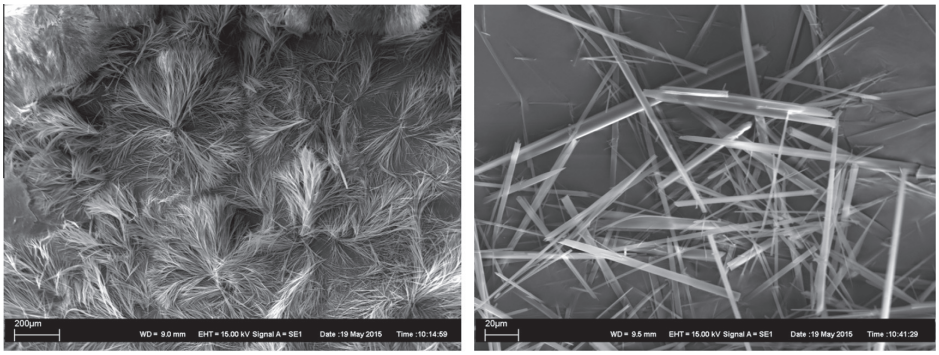


Fig. 3. 2% (w/v) gel of compound **1** in 50:50 H_2O:2-propanol. Sample prepared from gel (left) and sample prepared from hot solution (right).

Table 4
Toxicity of tested compounds evaluated using NR assay.

Compounds	Concentration range (μg/ml)	IC_{50} ± S.D. (μg/ml)
1	0.2–25	22.70 ± 2.16
2	0.2–25	12.31 ± 2.08
3	0.8–100	73.50 ± 4.58
4	0.8–100	*65.17 ± 3.30

* Cell viability evaluated at the highest concentration express as % of control.

the single fibers were longer, the length varied between 60 and 410 μm. The diameter of the spherical assemblies varied between 390 and 740 μm.

3.5. Results of the neutral red assay

The standard mouse cell line Balb/c 3T3, commonly used in accredited tests, was used for the toxicity study. The neutral red (NR) assay was performed for compounds **1–4** (see ESI: Eq. E1 and Figs. S1–S4). It is based on the fact that live (non-damaged) cells intake and store NR into their lysosomes, and the concentration of the incorporated dye can then be determined spectrophotometrically. The toxicity data are expressed as IC_{50} ± S.D. (μg/ml) values. Substances efficiency in inhibiting specific biochemical or biological function is described as the half maximal inhibitory concentration (IC_{50}). As can be seen from Table 4, the highest cell viability in the series of compounds was shown by compound **3**, which did not form any gels. Compound **1**, which formed hydrogels, showed third lowest toxicity in the series. In a previous study performed by us for bile acid-cysteamine conjugates [35] it was observed that the hydroxyl group in position 7α and/or 12α might cause the compound to show toxicity, whereas the non-toxic compounds only had a 3α-hydroxyl, 3α- and 7β-hydroxyls, or no hydroxyl groups at all. The current results do not agree with those hypotheses, even though compound **4** bearing no hydroxyls shows the second lowest toxicity in the series.

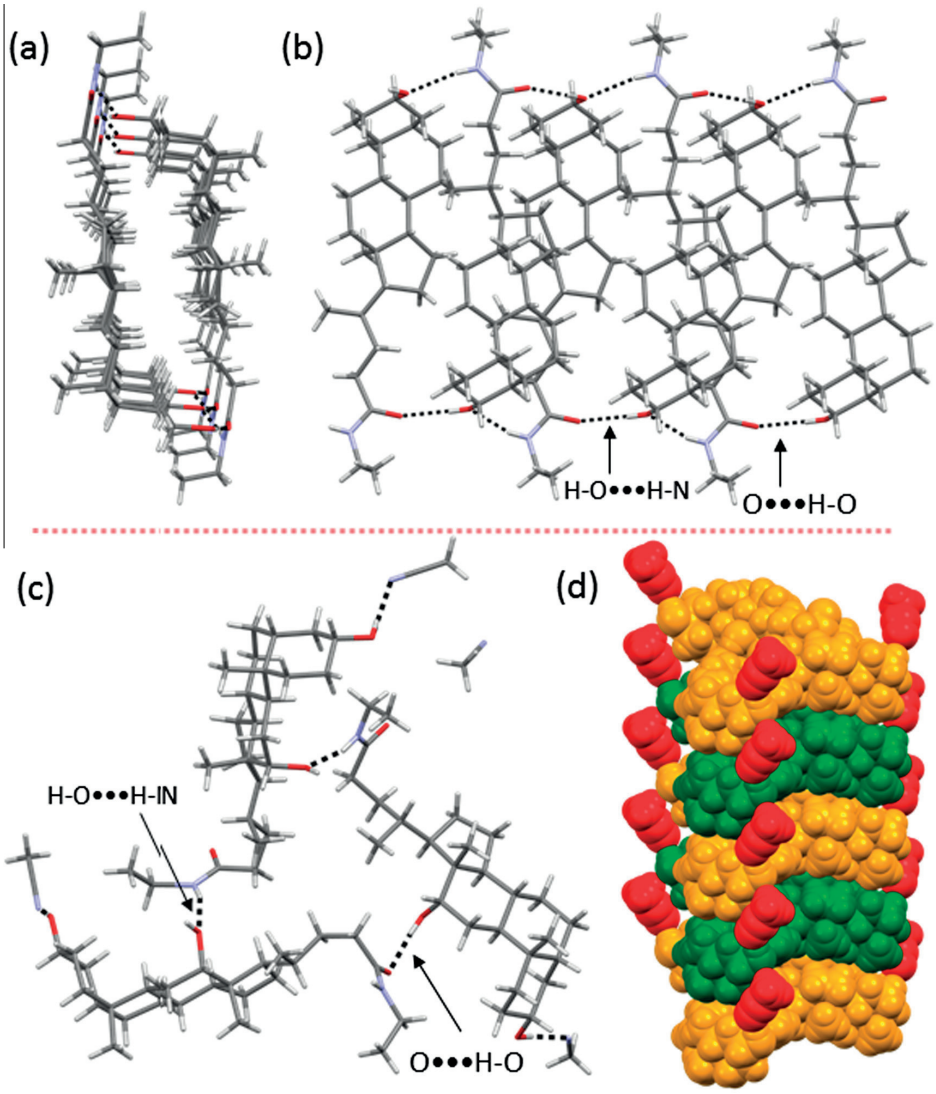


Fig. 4. (a) 1-D polymeric structure of **1**, and (b) side-view to show 3α-hydroxyl group and *N*-ethylamide chain orientation in capped stick model. (c) Top-view of 1-D hydrogen bonded trimeric structure of **2** in capped stick model. (d) Side-view to stacking arrangement of trimers in **2** in CPK model. Representation: orange and green – compound **2**, and red – acetonitrile. Black broken lines represent hydrogen bonding.

Table 5
Dihedral angles of the side-chains (°) of **1**, **2**, and **3**.

	1	2	3
C13–C17–C20–C22	174.3(2)	−174.0(2)	175.3(3) [169.5(3)]
C17–C20–C22–C23	−173.5(2)	−174.0(2)	59.4(4) [−156.9(3)]
C20–C22–C23–C24	172.6(2)	72.3(3)	178.9(3) [−163.8(4)]
C22–C23–C24–N24	136.3(2)	−125.9(3)	−3.6(5) [−155.0(4)]

3.6. X-ray crystallography

Despite many bile acid crystal structures have characteristic bilayer hydrogen bonding, the position and number of hydroxyl groups on the amphiphilic face play an important role to form a variety of hydrogen bonded supramolecular networks. These robust facial amphiphiles with different number of hydroxyl groups are carefully engineered for different applications *e.g.* micellar and interactions with biological membranes [47–49]. Another appealing solid-state property, which leads to remarkable lattice stabilization patterns, is their side chain modification. Modifications of side chains in bile acids have been extensively reviewed [50–54] and unarguably the conformations adopted by side-chains has represented wide range of applications in the field of crystal engineering, co-crystals, improved biological activity, and pharmaceutical applications. In the current study, inspired by the gelation properties, single crystals of *N*-ethylamide derivatives of lithocholic acid (**1**), deoxycholic acid (**2**) and cholic acid (**3**) were grown from acetonitrile by solvent evaporation to study

the effect of inclusion of amide functionality to the hydrogen bonded bilayers.

Compound **1** crystallized in the monoclinic space group $P2_1$ with one molecule in the asymmetric unit. As can be seen from Fig. 4a the peripheral hydroxyl group at 3α-position and the *N*-alkylamide side chain with *tttt* conformation [55] are orientated in the same direction. As a result, the hydroxyl group at 3α-position as well as the carbonyl and N–H groups in the side-chain drive **1** to give infinite one-dimensional bilayers stabilized by O–H···O [*d*(O···O), 2.857(3) Å] and N–H···O [*d*(N···O), 3.083(3) Å] interactions (Fig. 4b). Compound **2**, however, crystallized in the triclinic space group *P*-1 containing three crystallographically independent molecules of **2** (see Table S1) and four acetonitrile molecules in the asymmetric unit. The dihedral angles C13–C17–C20–C22, C17–C20–C22–C23, C20–C22–C23–C24, and C22–C23–C24–N24 (Table 5) for all the three molecules in the asymmetric unit are close to *ttgt* conformations. The hydroxyl groups at 12α-positions, the carbonyl oxygens, and the N–H groups form intermolecular O–H···O and N–H···O hydrogen bonds while the peripheral hydroxyl groups at 3α-positions hydrogen bonds to acetonitrile molecules (Fig. 4c). Although the molecule has features to crystallize in high symmetry space groups with screw axes, the hydroxyl groups at 12α-position and the fourth acetonitrile molecule presumably destroy the symmetry to bring molecules closer to form O–H···O and N–H···O interactions giving **2** wriggly motifs. The intermolecular hydrogen bond between the three bile acid molecules contributes to the induction of a 1-D columnar type assembly, possessing analogous features of helices, as shown in Fig. 4d. A perspective view of the crystal packing in **2** suggests that the self-organization in bile acids is dependent on positions of the hydroxyl groups and on the side chain conformations.

Compound **3** crystallized in the monoclinic space group *C*2, the asymmetric unit containing two crystallographically independent molecules of **3** and an acetonitrile molecule. The structural difference in the side chains with *tgtg* and *tttt* conformations, and the intermolecular N–H···O connectivity resulted in twice the unit cell *a*-axis length of **1**, as shown in Fig. 5a. The side chain with the *tgtg* conformation carrying an N–H group, and the hydroxyl groups of amphiphilic face of compound **3** give a bilayer structure by O–H···O and O–H···O interactions. On the other hand, the side chain with *tttt* conformation carrying the N–H group hydrogen bonds directly to an acetonitrile molecule as shown in Fig. 5b.

The bilayered structures formed by O–H···O interactions are robust, and the structural rigidity gives the lipophilic faces a characteristic corrugated property. The repulsion between methyl groups in the crystal packing makes the lipophilic face offset stabilized by hydrogen bonding and van der Waals interactions. As a result, based on the interdigitation of C18 and C19 methyl groups in lipophilic face and two possible conformations of the side chain, the host frameworks in these crystal structures were classified into four types; α-*gauche*, β-*trans*, β-*gauche*, and α-*trans* [56]. The side chain dihedral angle (C17–C20–C22–C23) is generally used to distinguish the four types. The side chain with *tgtg* conformation has dihedral angle (C17–C20–C22–C23) of 59.4(4)°, which corresponds to α-*gauche* type, whereas the interdigitation of methyl group suggests the host-framework being of β-*gauche* type. On the other hand, the side chain with *tttt* conformation has a dihedral angle (C17–C20–C22–C23) of −156.9(3)°, in which the stacking associates uniquely to α-*trans* type, as shown in Fig. 6. To the best of our knowledge, two host framework arrangements identified within a crystal packing has not been previously reported.

During crystallizations, most bile acids prefer anhydrous form due to bilayered O–H···O interactions, and thus can adopt significant conformational changes for side chains in co-crystals. Bile acids without hydroxyl groups are often and sometimes difficult to crystallize. For example in the current study, attempts to obtain

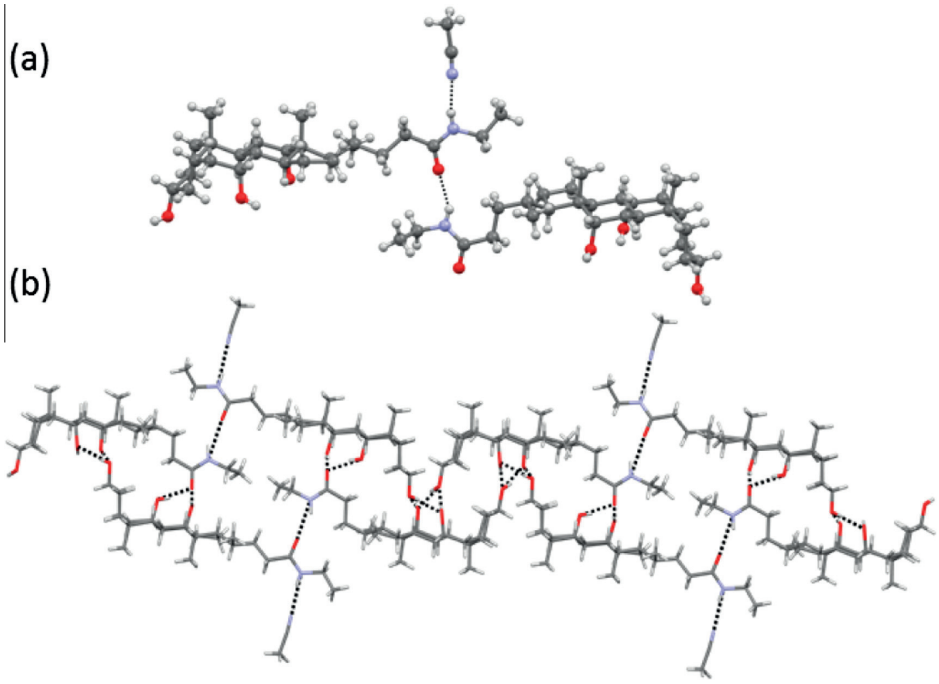


Fig. 5. (a) Asymmetric unit of **3** in ball and stick model, and (b) section of crystal packing to show bilayer formation (capped stick model). Hydrogen and solvent molecules are omitted for clarity. Black broken lines represent hydrogen bonding.

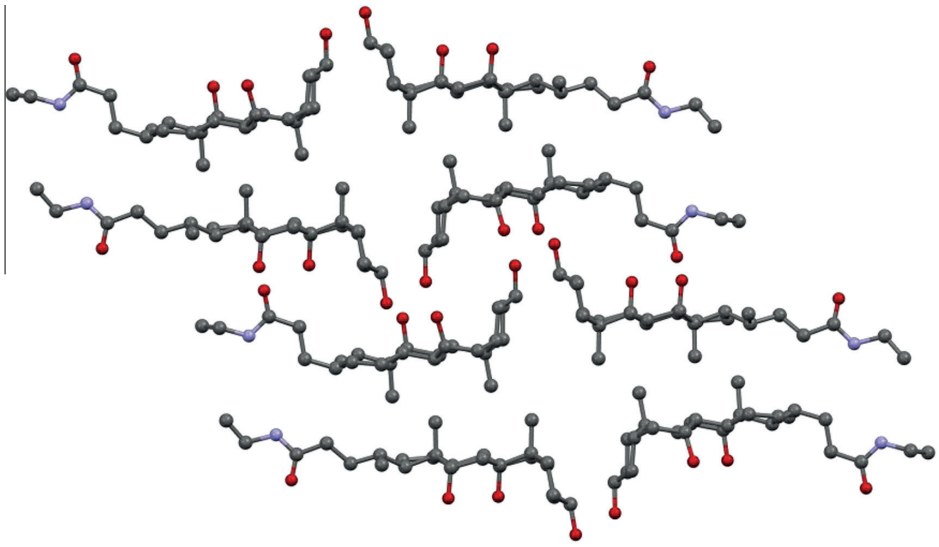


Fig. 6. Section of crystal packing viewed along *b*-axis to show α-*gauche* and α-*trans* type host arrangements in **3**, in ball and stick model. Hydrogen and solvent molecules are omitted for clarity.

good quality crystals of compound **4** were unsuccessful. Thin needle shape crystals were obtained after boiling **4** overnight in 1:1 water:methanol solvent ratio (data completeness <IUCr limit). However, the crystal structure shows that the lattice stabilization occurs uniquely at N–H functional group with methanol and water molecules.

Crystal data and X-ray experimental details for compounds **1**, **2**, and **3** are presented in Table S2.

4. Conclusions

As a continuation of the series of bile acid alkyl or functionalized alkyl amide/ester derivatives capable of acting as gelators, in this work four new bile acid ethyl amide derivatives were synthesized and characterized in detail. Gelation experiments for the synthesized molecules resulted in a total of 19 gel systems, most of which in aromatic solvents. Lithocholic acid derivative **1** formed the majority of the gel systems, which is common for bile acid derivatives. Furthermore, compound **1** generated five hydrogel systems, which showed thixotropic nature. For the best of our knowledge, this is the first time when supramolecular hydrogels involving bile acid alkyl amides is reported. The properties of the hydrogels were investigated using SEM and IR spectroscopy, and the toxicity of the compounds determined. Solid state packing of compounds **1**-**3** was defined by using single crystal X-ray crystallography. The packing patterns may provide an idea about the gel state assembly of the molecules, eventually shedding light to the phenomena underlying gel formation.

Bile acid-based hydrogels have potential use in biological applications both because they form in biocompatible solvents and because they are formed by nontoxic endogenous gelators. The biocompatibility of a hydrogel provides perhaps new potential applications regarding biomedicine, such as drug delivery, artificial tissue engineering, etc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2016.02.014.

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II

SYSTEMATIC MODULATION OF THE SUPRAMOLECULAR GELATION PROPERTIES OF BILE ACID ALKYL AMIDES

by

Riikka Kuosmanen, Rakesh Puttreddy, Kari Rissanen & Elina Sievänen, 2018

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FULL PAPER

Systematic Modulation of the Supramolecular Gelation Properties of Bile Acid Alkyl Amides

Riikka Kuosmanen,[a] Rakesh Puttreddy,[a] Kari Rissanen*[a] and Elina Sievänen*[a]

Abstract: The self-assembly properties of nine bile acid alkyl amide-based low-molecular weight gelators (LMWGs) are studied in detail. Based on the results the number of hydroxyl groups attached to the steroidal backbone plays a major role in the gelation, although the nature of the aliphatic side chain modulates the gelation abilities as well. Of the 50 gel systems studied, 35 are based on lithocholic acid and 15 on cholic acid derivatives. The deoxycholic acid derivatives did not form any gels. The gelation commences primarily in aromatic solvents and the gels manifest typical fibrous or spherical morphologies. The ^{13}C CPMAS NMR spectra measured from the crystalline materials and the corresponding wet organogels are analogous, suggesting that the chemical environments *viz.* the intermolecular interactions found in the organogels and in the crystalline state are similar. The single crystal X-ray structures of all nine bile acid amide derivatives studied revealed very similar molecular conformations in the solid state and gave insights into the possible intermolecular interactions in the gel state.

Introduction

Supramolecular gels formed by low molecular weight gelators (LMWGs) have been under intensive research in the past decades.[1–3] The properties of these soft systems differ from those of pure solids or liquids leading to emergence of applications in numerous fields, such as sensing,[4] biomedicine,[5] or materials technology.[6] Some of the recent examples include the use of supramolecular ionogels in non-covalent antibacterial coatings[7], in environmental remediation[8], and as radical scavengers[9]. Supramolecular gels manifest a network consisting of fibers or other nano- or microstructures, which immobilizes the bulk solvent by weak interactions, such as hydrogen bonding, π-π interactions, metal ion coordination, or van der Waals forces.[1,2] Some gel materials exhibit crystalline character, and the organization of the gelator in the crystalline state of the gel fiber can provide a concrete way of linking aspects of these two extremes. This lays the foundation for trying to understand how changes in the molecular structure affect the properties of the formed gels.[10-22]

Bile acids are end products of cholesterol metabolism formed in the liver enhancing the digestion and absorption of lipids and lipid-soluble vitamins. They are of pharmacological interest, being potential carriers and enhancers of absorption of drugs as well as major regulators of cholesterol homeostasis.[23–24] Bile acids possess a unique structure with a convex hydrophobic β-side, a concave hydrophilic α-side, and a polar side chain.[25] Because the molecule is polar on one side and apolar on the other, it is facially amphiphilic and its aggregates in water differ from those of classical surfactants.[26] Since the difference in the steric crowding enables derivatization of each hydroxyl group attached to the steroidal backbone individually, the water/lipid-solubility of the molecules can be easily tailored. More convertibility is achieved by modifying the side chain of bile acids.[27–28]

Several bile acid amide derivatives containing a functional group at the end of the aliphatic side chain have been reported and their properties studied by us.[29–36] Many of them have shown to be effective gelators, and one even formed stimuli-responsive metallogels.[34] Recently, we have focused on the bile acid derivatives that have no functional group at the end of the aliphatic side chain. The aim has been to determine the role of the functional group in self-assembly properties. Within the group of bile acid ethyl amides, the lithocholic acid derivative was shown to form hydrogels in addition to organogels.[37]

In the current study, the self-assembly and gelation properties of nine bile acid alkyl amides were studied in detail. Careful control of the crystallization under equal conditions resulted in good quality single crystals of all nine amides. The subsequent single crystal X-ray studies showed that the N···H, O···H, and H···H interactions were the most important intermolecular interactions affecting organization of the molecules in the solid state. Of the 50 gel systems formed, 35 consisted of lithocholic acid (**LCA**) derivatives and 15 of cholic acid (**CA**) derivatives, whereas deoxycholic acid (**DCA**) derivatives did not form any gels. Even though the length and branching of the aliphatic side chain clearly have an effect on the gelation abilities of the compounds, the number of hydroxyl groups attached to the steroidal backbone plays a major role in the gelation. The ^{13}C CPMAS NMR spectra measured from both the crystalline material and the corresponding wet gels are analogous, suggesting that the chemical environments *viz.* the intermolecular interactions found in the gel and in the crystalline state are similar.

Results and Discussion

Synthesis

LCA, **DCA**, and **CA** amides **LCA1**−**CA9** were synthesized by following straightforward and facile literature methods (Scheme

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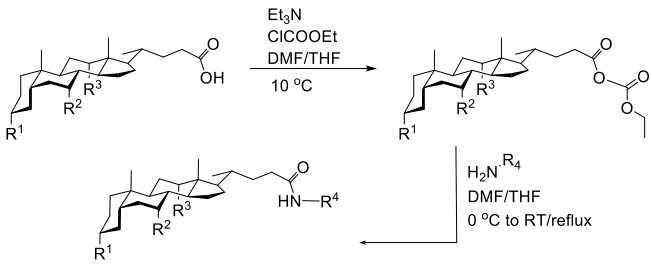
1).[29–30,32–34,37–38] All compounds were purified by simple recrystallizations with yields varying between 32 % and 73 % (See Electronic Supporting Information for more details).



LCA1 R^1=OH, R^2=H, R^3=H, R^4=$(CH_2)_2CH_3$
DCA2 R^1=OH, R^2=H, R^3=OH, R^4=$(CH_2)_2CH_3$
CA3 R^1=OH, R^2=OH, R^3=OH, R^4=$(CH_2)_2CH_3$
LCA4 R^1=OH, R^2=H, R^3=H, R^4=$(CH_2)_3CH_3$
DCA5 R^1=OH, R^2=H, R^3=OH, R^4=$(CH_2)_3CH_3$
CA6 R^1=OH, R^2=OH, R^3=OH, R^4=$(CH_2)_3CH_3$
LCA7 R^1=OH, R^2=H, R^3=H, R^4=$(CH_2)_2C(CH_3)_2$
DCA8 R^1=OH, R^2=H, R^3=OH, R^4=$(CH_2)_2C(CH_3)_2$
CA9 R^1=OH, R^2=OH, R^3=OH, R^4=$(CH_2)_2C(CH_3)_2$

Scheme 1. The synthetic route leading to compounds **LCA1**−**CA9**.

Single Crystal X-ray Analysis

Single crystals of compounds **LCA1**−**CA9** suitable for X-ray diffraction analysis were obtained by slow evaporation of either acetonitrile, DMF, or ethyl acetate solutions. The compounds create a pseudo isomorphous and isostructural series, crystallizing in the monoclinic space group $P2_1$ without solvent molecules (Figures 1 and 2). This differs markedly from the behavior of bile acid derivatives with shorter alkyl (ethyl) chains, where solvent molecules have a key role in stabilizing the crystal lattice.[37] This suggests that for the compounds **LCA1**−**CA9** with longer alkyl side chains the intermolecular bile acid-to-bile acid interactions are preferred over the bile acid-solvent interactions. The hydroxyl and amide groups are fully utilized in forming the intermolecular O–H···O and N–H···O hydrogen bonds to give a typical ordered 1-D bilayered structure (Figure S7).[39–41] The Hirshfeld surface analysis[42–45] for crystal structures of compounds **LCA1**−**CA9** indicates that these bilayers form a compact 3-D crystal lattice by high percentage of H···H interactions (Table S6 and Figures S9-S17).

Gelation Properties

The gelation abilities of compounds **LCA1**−**CA9** were tested in 36 solvents and of compounds **LCA1**−**CA6** additionally in 12 aqueous solutions. The results obtained are presented in Electronic Supporting Information (Tables S1-S3), while only selected gelation experiments are shown in Table 1. A total of 50 gel systems were formed, of which 35 by **LCA** and 15 by **CA** derivatives, respectively. In accordance with our previous results, **DCA** derivatives did not form any gels. Gel formation is mostly fa-

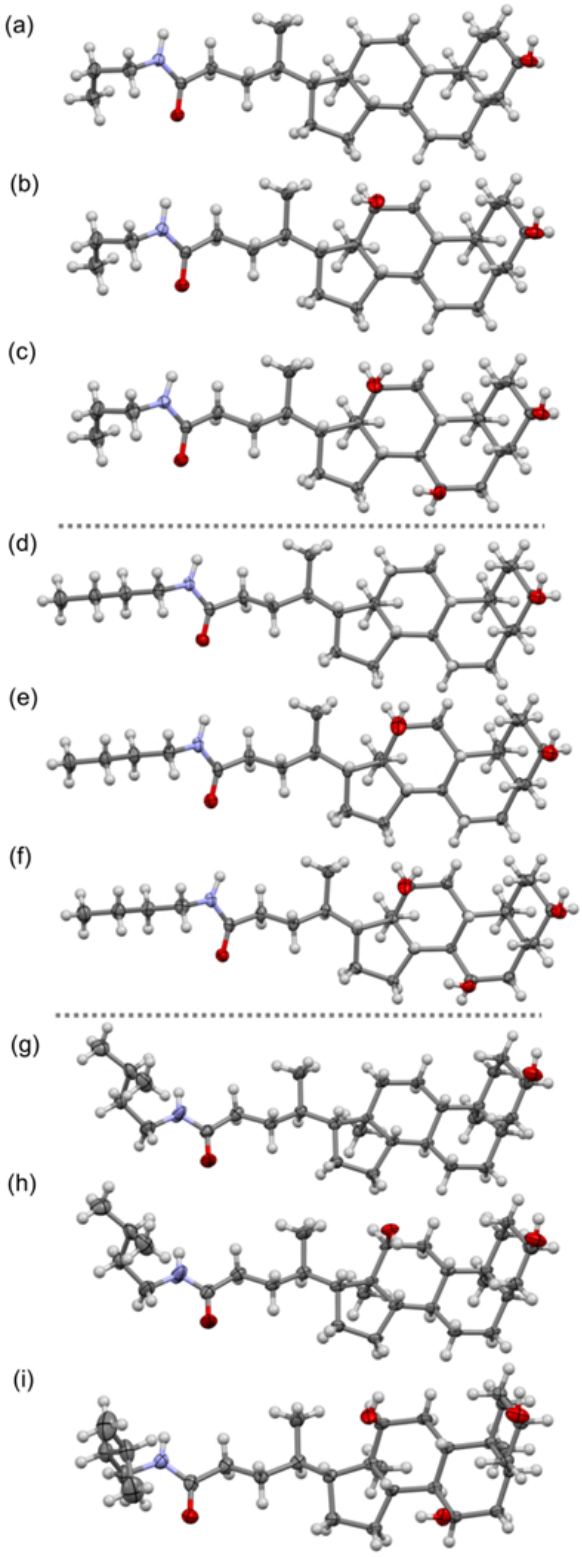


Figure 1. The X-ray crystal structures of (a) *N*-propyllithocholamide, **LCA1**, (b) *N*-propyldeoxycholamide, **DCA2**, (c) *N*-propylcholamide, **CA3**, (d) *N*-butyllithocholamide, **LCA4**, (e) *N*-butyldeoxycholamide, **DCA5**, (f) *N*-butylcholamide, **CA6**, (g) *N-iso*-pentyllithocholamide, **LCA7**, (h) *N-iso*-pentyldeoxycholamide, **DCA8**, and (i) *N-iso*-pentylcholamide, **CA9**, with thermal ellipsoids at 50% probability level.

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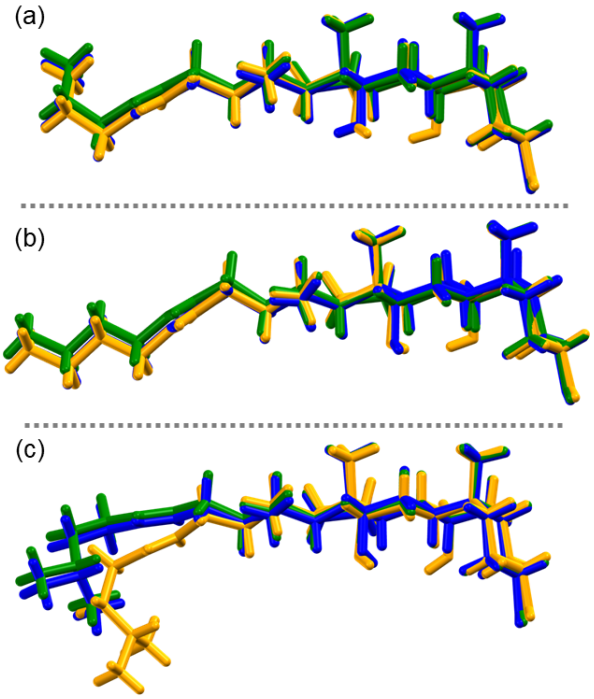

Figure 2. The overlay of the X-ray crystal structures of (a) **LCA1**, **DCA2**, and **CA3**, (b) **LCA4**, **DCA5**, and **CA6**, and (c) **LCA7**, **DCA8**, and **CA9** shown in capped stick model. Color codes: green (**LCA1**, **LCA4**, **LCA7**), blue (**DCA2**, **DCA5**, **DCA8**), and gold (**CA3**, **CA6**, **CA9**).

Table 1. Gelation test results at 1 % (w/v) for compounds **1**–**9**.

Solvent	LCA1	CA3	LCA4	CA6	LCA7	CA9
Benzene	NG	PG	**G**	NG	**G**, PG	**G**
Toluene	NG	**G-**	**G**	NG	**G-**	**G**
Ethylbenzene	NG	NG	PG	NG	**G**,**G-**, PG	**G**
o-Xylene	PG	NG	**G-**	**G-**	**G-**	**G**
m-Xylene	NG	NG	**G**	NG	**G**	**G**
p-Xylene	**G**	NG	**G**	NG	**G**	**G-**
tert-Butylbenzene	NG	NG	NG	NG	**G-**	PG
Cumene	**G-**,**G**	NG	**G**	NG	**G**	**G-**, PG
Chlorobenzene	**G**	NG	**G-**	NG	**G-**	NG
Anisole	**G**, PG	**G**	**G**	**G-** **G**	PG, **G-**	**G**
1,4-Dioxane	**G-**	**G-**	**G**	NG	**G-**, PG	PG
Acetonitrile	NG	NG	NG	NG	**G-**, PG	NG
Carbontetrachloride	**G-**, PG	NG	PG	NG	NG	NG
Ethyl acetate	**G-**, PG	NG	NG	NG	NG	NG

G = gel with appr. 0−5 % free solvent, **G-** = gel with appr. 5−20 % free solvent, PG = gel with appr. 50 % free solvent, NG = no gel.

vored in aromatic solvents, which is commonly observed for bile acid derivatives.[30–33,35–37] Furthermore, the gels of the lithocholic acid derivatives **LCA1**, **LCA4**, and **LCA7** as well as those of the cholic acid derivatives **CA3**, **CA6**, and **CA9** were thixotropic by nature similar to the gels formed by the ethyl amide derivatives reported previously by us.[37]

Because the formation of the bilayered structures, involving the functional groups in the head (hydroxyl groups) and tail (carboxylic acid groups or their derivatives) of the steroidal backbone, is typical for bile acid derivatives in the solid state,[39–41] it is reasonable to assume that similar structures exist also in the semi-solid or gel-states. In the case of **CA** and its derivatives the inter-bilayered interactions are reinforced by hydrogen bonds between the hydroxyl groups at positions 7α and 12α (Table S6). Thus the interstices between the bilayers formed up by the lipophilic β-faces of the steroidal backbones create suitable environments for the entrapment of aromatic solvent molecules. The **LCA** derivatives lack hydroxyl groups on the steroidal α-face, and are thus relatively lipophilic both on α- and β-sides. This property favors the entrapment of the aromatic solvent molecules. These structural features account for the tendency of **CA** and **LCA** derivatives to form supramolecular gels particularly in aromatic solvents. The **DCA** derivatives, which bear only one hydroxyl group on the α-side of the steroidal skeleton, manifest a different polarity profile. In addition, their mutual orientation is altered (Figures S21b and S22c), which prevents the formation of favorable space for entrapment of solvent molecules and thus disfavors supramolecular gel formation.

Morphology

The bile acid derivatives typically form gels consisting of differently organized fibres or of spherical assemblies. [30–33,35–37] The derivatives **LCA1**, **LCA4**, and **LCA7** formed gels with flat fibres in chlorobenzene (Figures 3a-c). The width of the fibres in the case of compounds **LCA1** and **LCA4** was approximately 600−800 nm and the length approximately 40−50 μm. Compound **LCA7** formed clearly thicker (up to 1.6 μm) and longer (200 μm) fibres. When the gelator concentration was increased from 1 % to 2 % (w/v), the fibres formed fan-shaped bundles. Similar fan-shaped bundles were observed previously within the 1 % hydrogels of **LCA** ethyl amide.[37] Interestingly, compounds **LCA1** and **LCA7** formed hollow nanotubes in ethyl acetate, as can be seen in Figure S3. The nanotubes of compound **LCA1** were approximately 390 μm long and 30 μm in diameter, whereas the flat fibres co-existing with the tubes were approximately 60 μm long and 950 nm wide, respectively. The nanotubes were clearly shorter and thinner in the case of compound **LCA7,** for which the length varied between 12 μm and 32 μm and the width between 700 nm and 3.3 μm.

The non-gel-forming amides **DCA2, DCA5**, and **DCA8** showed clearly crystalline nature. Compound **DCA2** formed a crystal with wood-like surface (Figure 3d), whereas rods with frayed ends were seen in the case of compound **DCA5** (Figure 3e). Compound **DCA8** possessed larger entities, which consisted of individual rods (Figure 3f). The largest assembly was approximately 19 μm long and 9 μm wide. The single rods were non-uniform in size; their lengths varied from 250 nm to 1 μm, whereas they were approximately 230 nm wide. Together with the

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entities consisting of rods extremely small spherical assemblies (1.8-3.3 µm in diameter) were observed in the case of compound **DCA8**.

The amides **CA3**, **CA6**, and **CA9**, for one, formed spherical assemblies, which coexisted with fibres. The assemblies of the propyl amide derivative **CA3** were spherical both in 1 % and 2 % chlorobenzene gels (Figure 3g). The 1 % gel consisted of spheres of 1.1−1.45 µm in diameter (Figure S1c), whereas in the 2 % gel the spheres were more versatile in size. The 1 % chlorobenzene gel of the butyl amide derivative **CA6** contained some crystalline material (Figure S1d). The crystals were rectangular in shape, and their sizes varied greatly; thickness varying between 21−67 µm and length between 40−230 µm, respectively. In the 2 % gel some of the crystals had further assembled in spherical structures (Figure 3h). The 1 % chlorobenzene gel of the *iso*-pentyl derivative **CA9** (Figure S4c) also consisted of spherical assemblies, whose diameters were considerably larger (120 µm) than those detected in the gel formed by compound **CA3** and the surface had a folded structure. The 2 % gel of compound **CA9** possessed an extensively folded surface (Figure 3i).

Based on the SEM images an obvious morphological difference between the gel-forming **LCA** and **CA** derivatives and the non-gel-forming **DCA** derivatives can be observed. The gel-forming derivatives possess fibrous and spherical structures, whereas the non-gel-forming derivatives form clearly crystalline assemblies.

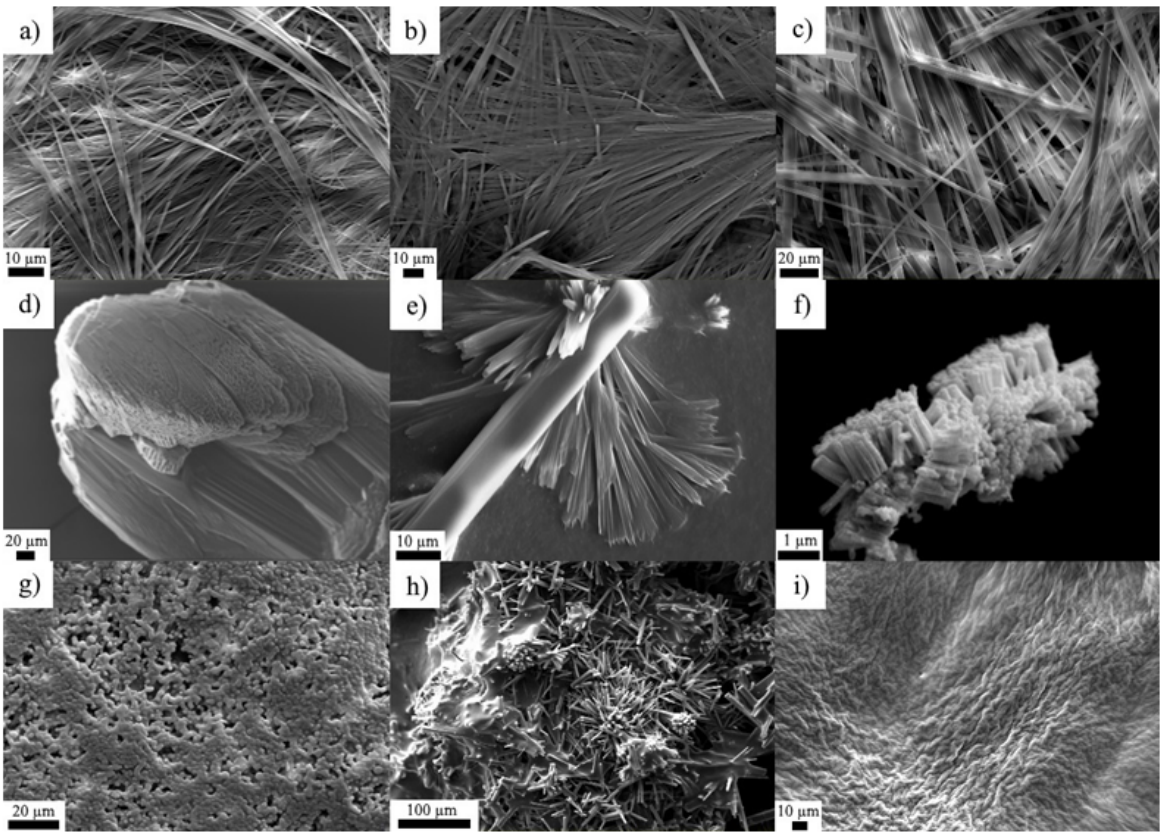

Figure 3. a) 2 % (w/v) chlorobenzene gel of compound **LCA1**, b) 2 % (w/v) chlorobenzene gel of compound **LCA4**, c) 2 % (w/v) chlorobenzene gel of compound **LCA7**, d) 2 % (w/v) chlorobenzene solution of compound **DCA2**, e) 2 % (w/v) chlorobenzene solution of compound **DCA5**, f) 2 % (w/v) chlorobenzene solution of compound **DCA8**, g) 2 % (w/v) chlorobenzene gel of compound **CA3**, h) 2 % (w/v) chlorobenzene gel of compound **CA6**, and i) 2 % (w/v) chlorobenzene gel of compound **CA9**.

^{13}C CPMAS NMR

For the ^{13}C CPMAS NMR measurements, 2 % (w/v) gel samples of compounds **LCA1**, **CA3**, **LCA4**, **CA6**, and **CA9** in chlorobenzene were prepared. Despite numerous efforts, for compound **LCA7** a reasonable S/N ratio was not reached by using a concentration of 2 % (w/v), which is why the concentration was increased to 4 % (w/v). The hot solution containing the compound in question was quickly pipetted into a rotor. The crystalline samples of compounds **LCA1**, **CA3**, **LCA4**, **CA6**, and **CA9** were prepared by recrystallizing the compounds in acetonitrile, and that of **LCA7** in DMF to maintain consistency with the single crystal X-ray analyses. The crystalline samples were dried under vacuum before the measurements. The gel samples were spun at a rate of 4 kHz and the crystalline ones at 10 kHz. The possibility of melting of the gel due to rotation was taken into

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account by using a slower spinning speed. Furthermore, the phase of the sample was ascertained to be a gel by visual inspection immediately after the measurement.

As can be seen from Figure 4, the ^{13}C CPMAS NMR spectra of the derivatives **LCA1**, **LCA4**, and **LCA7** measured from the crystalline materials corresponding to the X-ray crystal structures (Figure 1 and Figures 4a, c, and e) and from the chlorobenzene gels (Figures 4b, d, and f) are similar to each other. The same applies for the other gel-forming series, the derivatives **CA3**, **CA6**, and **CA9** (Figures S23-S25) as well. This suggests that the chemical environments and intermolecular interactions in the solid state and in the gel are alike.

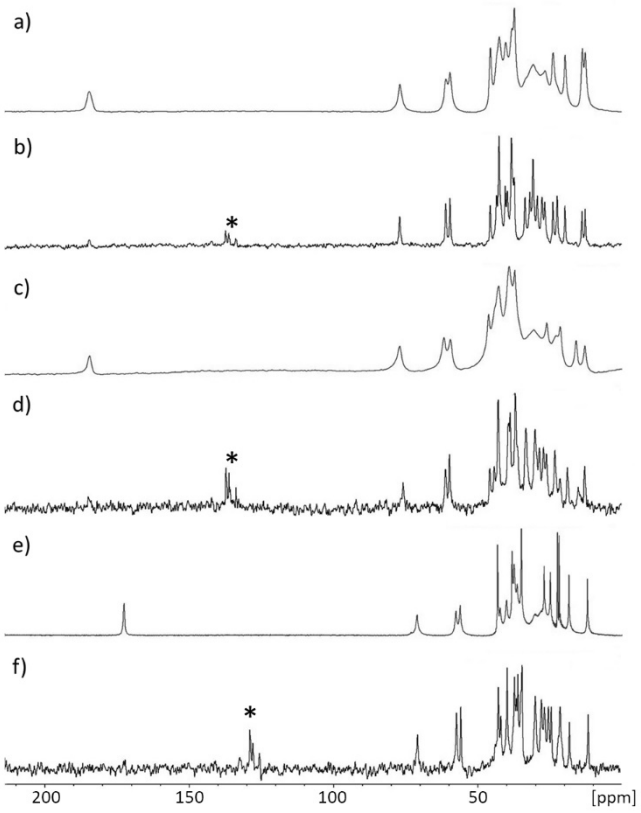

Figure 4. ^{13}C CPMAS NMR spectra of compounds **LCA1**, **LCA4**, and **LCA7**: **LCA1** recrystallized from acetonitrile (a), 2% (w/v) chlorobenzene gel of **LCA1** (b), **LCA4** recrystallized from acetonitrile (c), 2% (w/v) chlorobenzene gel of **LCA4** (d), **LCA7** recrystallized from DMF (e), and 4% (w/v) chlorobenzene gel of **LCA7** (f). In the gel samples, carbon signals from the chlorobenzene solvent are marked with an asterisk.

Conclusions

As a continuation of our systematic studies on the effect of the side chain modification on the gel-forming properties of bile acid derivatives, a detailed study on self-assembly properties of nine bile acid alkyl amides is reported. Based on the results the number of hydroxyl groups attached to the steroidal backbone seems to play a major role in gelation, although the nature of the aliphatic side chain has an impact on the gelation abilities as well. Of the 50 gel systems formed, 35 consisted of **LCA** and 15 of **CA** derivatives, whereas **DCA** derivatives did not form any gels. The intermediate polarity profile on the α-side of the **DCA** derivatives seems to prevent favourable interactions for supramolecular gel formation. The gels of **LCA** and **CA** derivatives were formed mostly in aromatic solvents exhibiting typical fibrous or spherical morphologies. The ^{13}C CPMAS NMR spectra measured from the crystalline materials corresponding to the X-ray crystal structures and from the chlorobenzene gels of **LCA** and **CA** derivatives are similar to each other indicating similar chemical environments and intermolecular interactions in the crystalline state and in the gels. When used with caution the correlation between single crystal X-ray crystallography and solid-state NMR may provide additional insight for inspecting the structure of the supramolecular gels.

Experimental Section

Details of the syntheses, compound characterizations, and gelation property studies are given in the Electronic Supporting Information.

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Keywords: bile acid amides • X-ray crystallography • supramolecular gels • intermolecular interactions • CPMAS NMR

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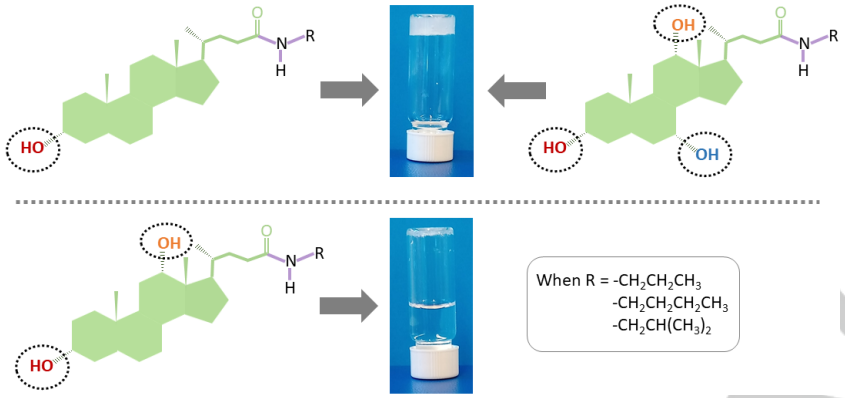

Riikka Kuosmanen, Rakesh Puttreddy, Kari Rissanen, and Elina Sievänen**

Page No. – Page No.

Systematic Modulation of the Supramolecular Gelation Properties of Bile Acid Alkyl Amides

A detailed study on the self-assembly properties of nine bile acid acid alkyl amide-based low-molecular weight gelators resulted in 50 gel systems, 35 of which were based on lithocholic acid and 15 on cholic acid derivatives. Deoxycholic acid derivatives, however, did not form any gels. Gelation commenced primarily in aromatic solvents and the gels manifested typical fibrous or spherical morphologies. The ^{13}C CPMAS NMR spectra measured from the crystalline materials and the corresponding organogels were analogous, suggesting that the chemical environments and consequently the intermolecular interactions in the organogels and in the crystalline state are similar. The single crystal X-ray structures of all nine bile acid amide derivatives revealed similar molecular conformations in the solid state giving insights into the possible intermolecular interactions in the gel state.

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III

THE EFFECT OF THE SIDE CHAIN ON GELATION PROPERTIES OF BILE ACID ALKYL AMIDES

by

Riikka Kuosmanen, Khai-Nghi Truong, Kari Rissanen & Elina Sievänen, 2021

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The Effect of the Side Chain on Gelation Properties of Bile Acid Alkyl Amides

Riikka T. Kuosmanen, Khai-Nghi Truong, Kari T. Rissanen,* and Elina I. Sievänen*[a]

Six bile acid alkyl amide derivatives were studied with respect to their gelation properties. The derivatives were composed of three different bile acids with hexyl or cyclohexyl side chains. The gelation behaviour of all six compounds were studied for 36 solvents with varying polarities. Gelation was observed mainly in aromatic solvents, which is characteristic for bile-acid-based low molecular weight gelators. Out of 108 bile acid-solvent combinations, a total of 44 gel systems were formed, 28 of which from lithocholic acid derivatives, only two from deoxycholic acid derivatives, and 14 from cholic acid derivatives. The majority of the gel systems were formed from bile acids with hexyl side chains, contrary to the cyclohexyl group, which seems to be a poor gelation moiety. These results indicate that the spatial demand of the side chain is the key feature for the gelation properties of the bile acid amides.

1. Introduction

Research on supramolecular gelation and the gel properties has tremendously increased over the past three decades. The field of supramolecular gels is intriguing because of the multitude of compounds[1,2] and mixtures of compounds[3] which are able to form gels. This ever-expanding library of compounds has provided many fascinating supramolecular gel systems with possible applications in regenerative medicine,[1,4–6] treatment of cancer,[1,7] environmental remediation,[8–12] imaging[1] as well as many others. The formation of the supramolecular gel occurs by immobilization of the solvent through weak interactions, such as hydrogen bonding, π-π-interactions, or metal coordination. A self-assembled network of fibres or other nano- or microstructures is spontaneously formed by the gelator molecules. Because both supramolecular gelation and crystallization arise from nucleation processes,[13] and some gelator molecules have a very strong tendency to form single-crystalline materials, the information obtained from solid-state structures via single crystal X-ray crystallography can shed light on the interactions responsible for the formation of the supramolecular gels. Detailed structural information offers a possibility to draw conclusions on how the structure of the gelator molecule correlates with the self-assembly, i.e. gelation properties. The impact of structure on gelation behaviour has been studied for many types of compounds such as divergent organic salts[14–16] and bile acid derivatives containing amino acid moieties.[17–18]

In the liver, cholesterol is converted to bile acids during complex biosynthetic paths.[19–22] Thus, because bile acids are intrinsic to human bodies, they are biologically and chemically interesting. Bile acids assist in the absorption of lipids and lipid soluble vitamins. On the other hand, defects in the bile acid metabolism or excretion can lead to severe medical conditions. In supramolecular chemistry, bile acids are intriguing starting materials, since they can be relatively easily modified, provide divergent steroidal backbones, and are cheap and easily available starting materials. Bile acids are also amphiphilic, containing hydrophilic and hydrophobic sides, from which the different self-assembly motifs together with chemical and biological properties arise. Hence, bile acids and their derivatives have multiple applications in medicine, for example in treatment of liver diseases[23,24] as well as for diabetes[25–28] and as antimicrobial agents.[29]

During the past fifteen years, a large number of bile acid derivatives have been studied regarding their gelation properties in our research group.[17,18,30–38] Recently, we have been interested in bile acid derivatives which do not have any functional group at their end of the side chain. In this respect, we wondered how the length and the shape (linear or branched) of the functional-group-free side chain affected the gel formation ability of a particular bile acid derivative.

Herein, we report six new bile acid derivatives, crystal structures for five of them, and the studies of their gelation properties. The compounds consist of three different bile acids with a hexyl- or a cyclohexylamide side chain. The gelation studies reveal that the side chain has a marked influence on the gelation ability of the bile acid amides. Clearly, the number of hydroxyl groups on the steroidal skeleton of the compound has an effect on gelation, verifying previous observations.[17,18,30–38]

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2. Results and Discussion

2.1. Synthesis

The bile acid amides **1**–**6** were synthesized through the route presented in Scheme 1, frequently utilized by our research group.[17,18,30–36] Compounds **1**–**6** were purified either by column chromatography or by recrystallization methods. The isolated yields varied from 11% to 54%. The syntheses and characterisation details for compounds **1**–**6** can be found in the Supporting Information.

2.2. Solid-State Structures

Single crystals of the amides **1**–**5** suitable for X-ray diffraction were obtained by slow evaporation of either their acetonitrile or 1,4-dioxane solutions. Unfortunately, not all of the crystallization attempts were successful. Despite numerous attempts, compound **6** was only obtained as a gel or amorphous powder. The solid-state structures of compounds **1**–**5** are depicted in Figure 1.

Compound **2** crystallized in the highly symmetrical tetragonal space group $P4_3$, whereas all the other hexyl and cyclohexyl derivatives, similar to the previously studied lithocholyl, deoxycholyl, and cholyl amides bearing ethyl,[34] propyl, butyl, and isopentyl side chains,[33] crystallize in the monoclinic space group $P2_1$.

As the previously studied ethyl, propyl, butyl, and isopentyl derivatives,[33,34] also the current series of hexyl- and cyclohexyl amides **1**–**5** crystallized without solvent molecules. Within the series of bile acid alkyl amides, bile acid-solvent interactions have hitherto only been observed for the *N*-ethyldeoxycholamide and *N*-ethylcholamide.[34] In addition to stabilizing the crystal lattice, the co-crystallized acetonitrile molecules had a significant influence on the structure of the side chain orientation of the respective bile acid, as can been seen in Figure 2 (red capped stick models).

In amides **1**, **2**, **4**, and **5**, intermolecular bile acid-bile acid interactions, i.e. O–H···O and N–H···O hydrogen bonds between hydroxyl and amide groups, are responsible for forming the typical ordered 1D bilayered structures (Figure 3(a) and Figures S8–S12).[33,39–41] Differing from the other derivatives, compound **3** forms a 2D hydrogen-bonded polymer where neighbouring 1D bilayered, tubular assemblies are interconnected through N–H···O hydrogen bonds as illustrated in Figure 3(c) – black broken lines. Since compound **3** crystallizes not with one molecule but two crystallographically independent molecules in the asymmetric unit, the *N*-alkyl side chains are oriented in different conformations (Figure S5) and are engaged in hydrogen bonding different to what had hitherto been observed.

The infinite 1D bilayer structure observed in virtually all bile acid amide structures[33,34] is stabilized by several intermolecular O–H···O and N–H···O interactions between the hydroxyl groups at the 3α(/7α/12α)-position(s) as well as by interactions between the carbonyl and N–H groups in the side chains of the bile acid amides. This known motif was observed for one of the

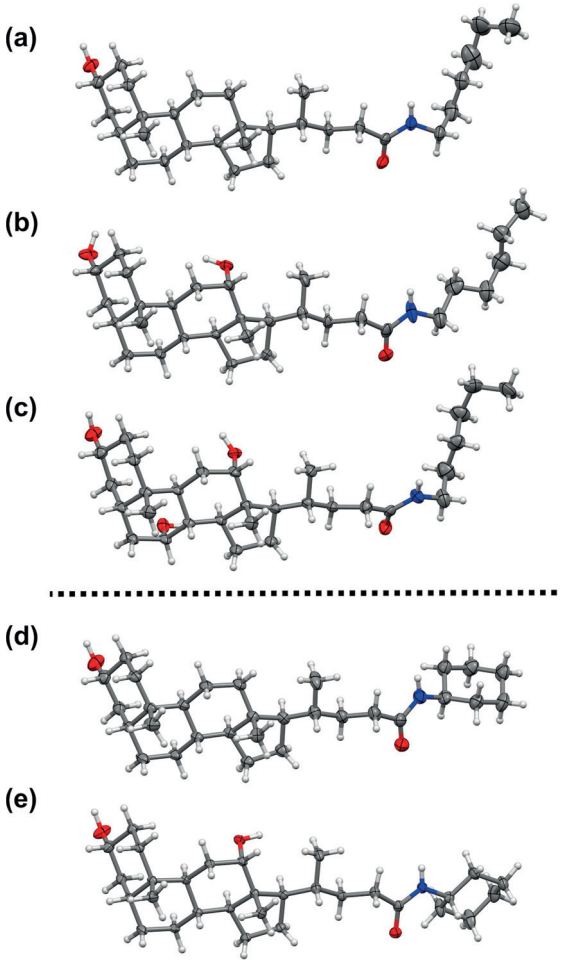
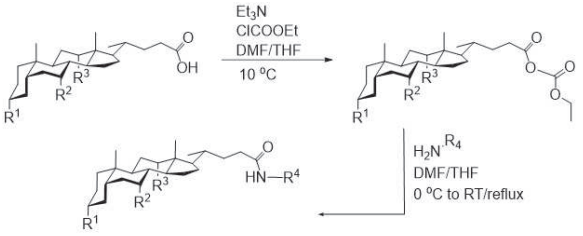


Figure 1. The X-ray structures of **1**–**5**: (a) *N*-hexyllithocholamide, **1**, (b) *N*-hexyldeoxycholamide, **2**, (c) *N*-hexylcholamide, **3**, (d) *N*-cyclohexyllithocholamide, **4**, and (e) *N*-cyclohexyldeoxychol-amide, **5**. Displacement ellipsoids are drawn at the 50% probability level. Atom sites with minor occupancies have been omitted for clarity. Color code: grey (C), white (H), red (O), and blue (N).



1 R^1=OH, R^2=H, R^3=H, R^4=$(CH_2)_5CH_3$
2 R^1=OH, R^2=H, R^3=OH, R^4=$(CH_2)_5CH_3$
3 R^1=OH, R^2=OH, R^3=OH, R^4=$(CH_2)_5CH_3$
4 R^1=OH, R^2=H, R^3=H, R^4=C_6H_{11}
5 R^1=OH, R^2=H, R^3=OH, R^4=C_6H_{11}
6 R^1=OH, R^2=OH, R^3=OH, R^4=C_6H_{11}

Scheme 1. The synthetic route to bile acid derivatives **1**–**6**.

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molecules of **3**. The other molecule in the X-ray structure of **3** forms the bilayered structures only with O–H···O hydrogen bonds. The "free" N–H groups act as linkers between the bilayers and are stabilized by N–H···O(carbonyl) interactions with d(N···O) = 3.008 Å. The polar and apolar layers alternate along the *b*-axis (Figure 3(d)).

Compared to the previously studied ethyl, propyl, butyl, and isopentyl derivatives,[33,34] the *n*-hexyl and cyclohexyl groups in compounds **1**–**5** impart flexibility with high degrees of conformational freedom (Figures S4 and S5). In addition, the flexibility of the alkyl chains increases with the number of hydroxyl groups attached to the steroidal backbone (Figure 2). Hirshfeld surface analyses[42–45] for the crystal structures indicate that the bilayers form a compact 3D crystal lattice through a high percentage of H···H contacts (Table S4 and Figures S13–17, Supporting Information). Surprisingly, the highest percentage of H···H contacts was observed for **2**, *N*-hexyldeoxycholamide, which contrasts the previously observed behavior where the H···H interactions decrease with an increasing number of hydroxyl groups attached to the steroidal backbone (Table S4). The X-ray structures of *N*-hexyldeoxycholamide, **2**, and *N*-cyclohexyldeoxycholamide, **5**, do not offer any apparent molecular or intermolecular-interaction-based explanation (see Hirshfeld surface analysis, Table S4) on not forming gels similarly to the OH-group-containing lithocholamides and the three OH-group-containing hexylcholamides (Table 1). Despite very similar molecular conformations and packing in the crystal lattice (Figures 1 and 2), the likely cause for the non-gelation is either too low or too high solubility in the target solvent.

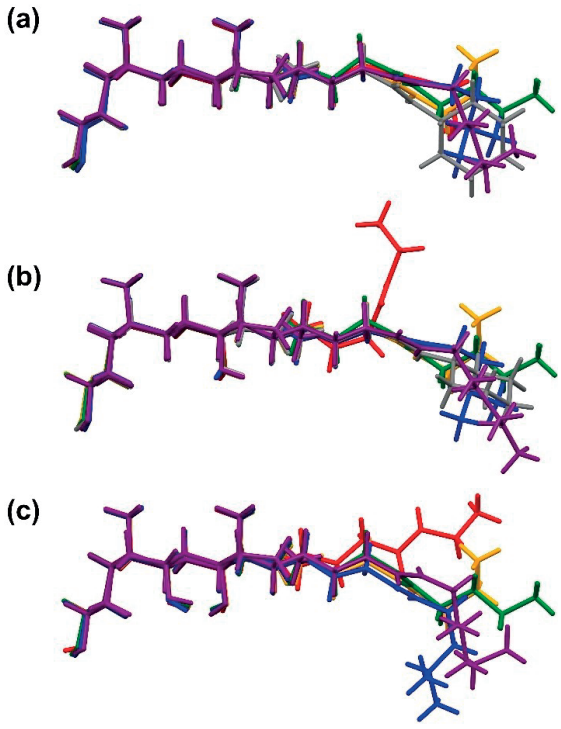

Figure 2. Overlay of the X-ray crystal structures of all (a) lithocholamide, (b) deoxycholamide, and (c) cholamide derivatives shown in capped stick model. Color code: red (*N*-ethyl),[34] gold (*N*-propyl),[33] green (*N*-butyl),[33] blue (*N*-isopentyl),[33] grey (*N*-cyclohexyl), and purple (*N*-hexyl). Co-crystallized solvent molecules as well as atom sites with minor occupancies have been omitted for clarity.

2.3. Gelation Studies

The results of the gelation test of compounds **1**–**6** conducted in 36 solvents were in good congruence with the results obtained previously in our group.[17,18,30–36] Most of the gel systems were formed in aromatic solvents and are presented in Table 1. More detailed information on the gelation experiments can be found in the Supporting Information. A total of 44 gel systems were formed, 28 of which by lithocholic acid derivatives (compounds **1** and **4**), two by a deoxycholic acid derivative (compound **2**), and 14 by cholic acid derivatives (compounds **3** and **6**). No gelation was observed in alcohols or chlorinated solvents with the exception of chlorobenzene.

The deoxycholic acid derivative **5**, containing a cyclohexyl side chain, did not form any gels. The other deoxycholic acid derivative with the hexyl side chain (compound **2**), however, formed two gel systems. This was unexpected, since deoxycholic acid derivatives do not usually self-assemble into gels as previously observed.[17,18,30–36] The majority of the gel systems were formed by lithocholic acid derivatives **1** and **4**. Compound **1** was observed to form gel systems in the largest number of solvents. The cholic acid derivative **3** was the only compound exclusively forming flawless gels, half of which started to degrade after four hours. According to our previous studies,[33,34] lithocholic acid derivatives have been more efficient gelator molecules, whereas in this study, mostly partial gels were obtained. For example, in the case of compound **1** which

Table 1. Solvents in which compounds **1**–**6** formed gel systems.

Solvent	1	2	3	4	5	6
Benzene	**PG**	**PG^s**	**G^f**	–	–	**G^f**
Toluene	**G^f**	–	**G^f**	**PG^s**	–	**PG^s**
Ethylbenzene	**PG**	–	–	**PG**	–	**G^s**
o-Xylene	**PG**	–	**G^f**	**PG**	–	**G^{es}**
m-Xylene	**G^f**	–	–	**PG**	–	**PG^s**
p-Xylene	**G^f**	–	–	**PG**	–	**PG^s**
Mesitylene	**PG**	–	–	–	–	–
tert-Butylbenzene	**G^f**	–	**G^f**	**PG**	–	–
Cumene	**PG**	–	–	**PG**	–	–
Chlorobenzene	–	–	**G^s**	–	–	**G^{es}**
Anisole	**PG^s**	–	**G^s**	–	–	**G^{es}**
ACN	**G^f**	–	–	–	–	–
Ethyl acetate	**G^f**	–	–	**PG**	–	–
n-Hexane	**G^f**	–	–	**PG**	–	–
Diethyl ether	**PG^f**	–	–	–	–	–
DMSO	–	–	–	**PG**	–	–
Formamide	–	–	–	**PG**	–	–
Ethylene glycol	–	**G^s**	–	**PG**	–	–
Cyclohexene	**PG**	–	–	**G**	–	–

Abbreviations: **G^f** (gel formed under 30 min), **G** (gel formed within an hour), **G^s** (gel formed after 1 day), **G^{es}** (gel formed extremely slowly, after months), **PG^f** (partial gel formed in 30 min), **PG** (partial gels formed within an hour), **PG^s** (partial gel formed after 1 day), and "–" (no gel).

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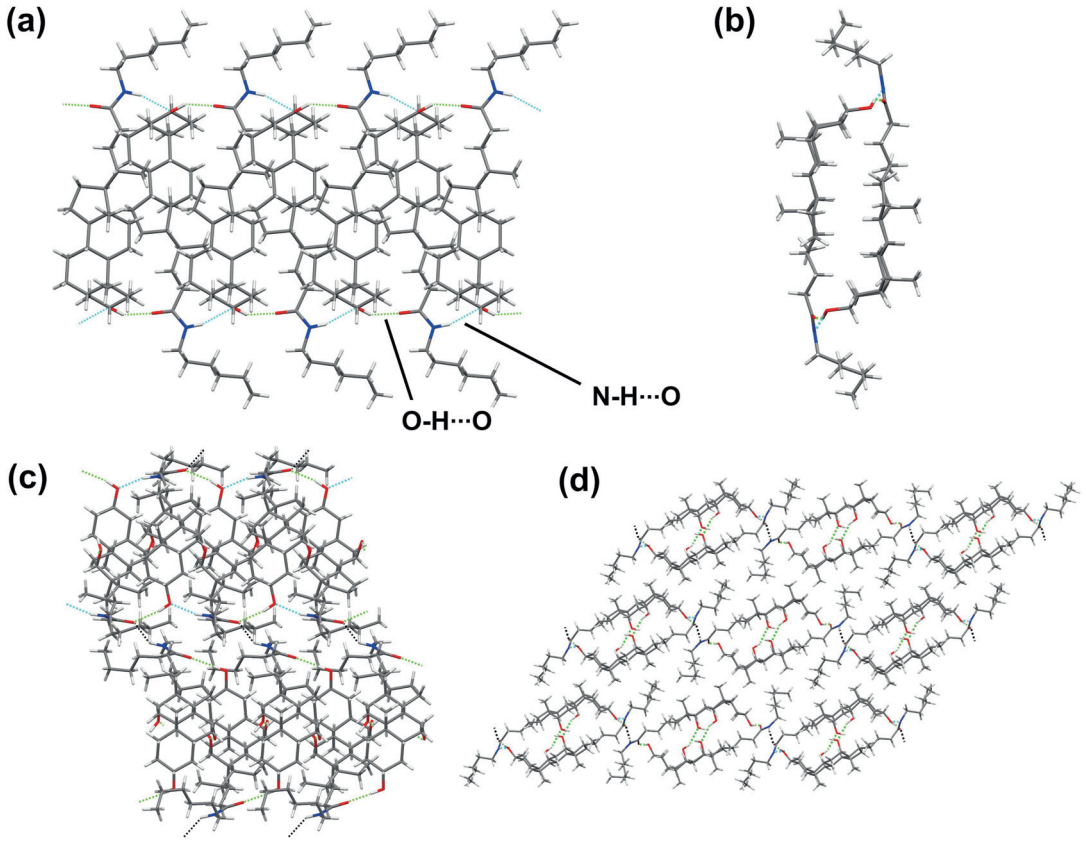

Figure 3. The 1D hydrogen-bonded polymer viewed along the *a*-axis (a) and the tubular assembly viewed along the *b*-axis of **1** (b), 2D hydrogen-bonded polymer viewed along the *a*-axis (c) and along the *b*-axis of **3** (d) in capped stick model. Atom sites with minor occupancies have been omitted for clarity. Broken lines represent N–H·O (turquoise and black) and O–H·O (green) hydrogen bonding. Color code: grey (C), white (H), red (O), and blue (N).

formed the largest number of gel systems, eight of the fifteen gels formed were partial.

The time of the gel formation was exceptionally short for compound **3** in most of the solvents: four out of the six gels formed in only three minutes. On the other hand, in chlorobenzene and anisole, the gel formation took four days. There appears to be a correlation between the temporal stability of the gel and the formation time: slowly formed gels were stable, whereas extremely quickly formed gels started to collapse within four hours (except the gel in *tert*-butylbenzene). A similar phenomenon was not observed with compound **6**, where both the quickly and the slowly formed gels were equally stable. Compound **1** self-assembled into gels fast as well, but the gelation time was considerably slower (over fifteen minutes) when compared with compound **3**. When comparing the melting points of the benzene gels of compounds **1**, **2**, **3**, and **6**, differences were observed. The benzene gels of compounds **1**–**3** had similar melting points ranging from 64 °C to 69 °C. Hence, the compound or the formation time of the gel did not have a pronounced effect on the melting point. However, the benzene gel of compound **6** deviated from this trend as it had a significantly higher melting point (80 °C). In the case of compound **6**, melting points of the gels were also determined in differing solvents. The gels in chlorobenzene, *o*-xylene and anisole exhibited lower melting points ranging from 70 °C to 76 °C than those observed for the corresponding benzene gel. The highest melting point was observed for the ethylbenzene gel of compound **6** (94 °C).

When considering the melting points of the gels in different solvents and the formation time of the gels, the least stable ones corresponded to the most slowly formed gels.

An interesting congruence with respect to the gelator molecule and the solvent was observed, showing that the lithocholic acid derivatives (**1** and **4**) formed gels when having a complimentary structure of the side chain and the solvent. Compound **1**, with its linear hexyl side chain, formed a gel in *n*-hexane, whereas compound **4** only formed a partial gel. In cyclohexene, compound **4**, with its cyclohexyl side chain, formed a gel and compound **1** self-assembled into a partial gel.

Differences in the appearance and consistence of the gel systems in different solvents were also observed. Compound **1** produced cleavable, bright gels in benzene and cyclohexene, whereas the gels in acetonitrile and diethyl ether were clearly fibrous. The gel of compound **4** in toluene was also fibrous.

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Gels in cumene were constructed of small spheres in the cases of compounds **1** and **4**. In addition, the gel of compound **4** in cyclohexene also consisted of spheres. The gels of compound **2** in benzene and ethylene glycol were ductile, as was the gel of compound **3** in benzene. Especially in the case of compound **3** in benzene, the gel appeared extremely stretchy. The gel formed by compound **6** in benzene, for one, resembled a jelly. All the gel systems were qualitatively stable; each either maintaining its shape or returning to a smooth-surfaced gel after being disturbed with a spatula. This was surprising, since in our previous studies the gels collapsed immediately when disturbed.[33,34]

Similar to our previous studies,[33,34] all the gels in the current study were thixotropic by nature.

Kamlet-Taft solvent parameters can be used in determining the solvents' properties.[46–48] They include three parameters that can be utilized in the analysis of solvent interactions in gel systems. The α- and β-parameters describe the hydrogen-bond-donating and accepting ability of the solvent, respectively. The third parameter, the π*-parameter, refers to the polarizability of the solvent. In the current study, all solvents in which gels or partial gels were formed were analyzed with respect of the three Kamlet-Taft parameters (see Figure 4). The gelating and non-gelating solvents are divided by a vertical line in Figure 4.

In our previous studies,[36] the α-parameter (hydrogen bond donor ability) was observed to be an important Kamlet-Taft parameter effecting the immobilization of the solvent by a gelating compound. The value was zero for most of the gel-forming solvents. In this study, the α-parameter exhibited similar properties (Figure 1) as it was zero for all the gel-forming solvents, with the exception of acetonitrile (0.19), formamide (0.71), and ethylene glycol (0.90). The majority of the β-parameter values (hydrogen bond acceptor ability) were in accordance with previous studies of bile acid derivatives having moderate values from 0.10 to 0.31.[34] The gelating solvents diethyl ether, ethyl acetate, dimethyl sulphoxide, and ethylene glycol have higher β-parameter values (0.47, 0,45, 0.76, and 0.52, respectively). This differs from our previous results,[36] where a higher β-value seemed to completely prevent gelation. The lack of the functional group at the end of the aliphatic side chain probably enables the gel formation in a larger versatility of solvents. On the other hand, the steroidal backbone may also play an important role: in the current study, lithocholic acid, deoxycholic acid, and cholic acid backbones were used, whereas in the previous one,[36] instead of cholic acid, chenodeoxycholic acid was used. When comparing the π*-parameter (polarizability) values, acetonitrile, formamide, and ethylene glycol again stand out with significantly higher values, as do chlorobenzene and anisole. For all the other gelating solvents, the value of the π*-parameter varies between 0 and 0.56.

When looking into the Kamlet-Taft parameters regarding the chlorinated solvents, an interesting difference is observed. Chlorobenzene, in which gels were formed, is clearly divergent from chloroform and dichloromethane, which did not induce gel formation. Chloroform and dichloromethane both possess a slightly higher hydrogen bond donor ability (α-value) than chlorobenzene. The hydrogen bond acceptor ability (β-value), however, seems to be a more significant factor for gelation within chlorinated solvents used in this study, since chlorobenzene has the highest value of the three. The same observation applies to the polarizability of the solvent (π*-value), which means that the solvation of peripheral groups of the gelator molecule and the fiber-fiber interactions play a major role in gel formation in chlorinated solvents.

When comparing the aromatic solvents in which gels were formed (excluding chlorobenzene), anisole seems different from the other aromatic solvents at first glance. Anisole has markedly higher β- and π*-values than other the aromatic solvents, but the α value is zero, as is the case with all the other aromatic solvents. When the ratio of β/π* is calculated (Table S2, Supporting Information), all aromatic solvents, however, show similar ratios around 0.30.

Compounds **1**–**6** were highly soluble in alcohols, meaning that gelation was not observed. All Kamlet-Taft parameters are,

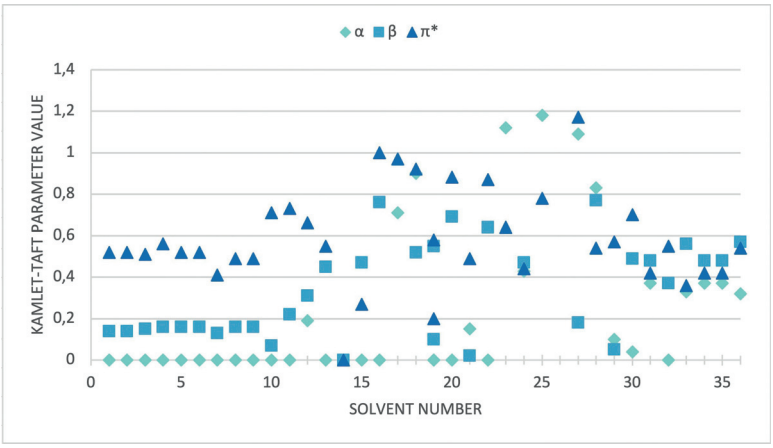

Figure 4. Kamlet-Taft parameters (α, β, and π*) for gel-forming (1–19) and not gel-forming (20–36) solvents (Table 1 and Supporting Information). The vertical line divides the gel-forming and the non-gel-forming solvents.

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in general, in the same range for the alcohols, which leads to the conclusion that solvents which possess relatively equal hydrogen bond donor and acceptor abilities are not good solvents for gelation with bile acid amides.

2.4. Morphology

In the SEM images of the selected dried gel systems (xerogels), mostly fibrous structures were seen. In the fibrous gel networks, the larger fibres were observed to have formed from thinner fibres (Figure S2).

The gels of compounds **3** and **6** formed in benzene were divergent: smooth ball-shaped structures were observed (Figure 5(a) and (b), respectively). For compound **3**, the diameter of the spheres varied from 1.3 μm to 3.9 μm. In the case of compound **6**, the spheres were fused https://www.merriam-webster.com/dictionary/elided and the gel appeared as dough-like in the SEM images. The spherical structures were uniform in size, the average diameter being 665 nm. The gel of compound **3** in benzene was extraordinary by its physical appearance also, because the gel was very elastic and stretchy.

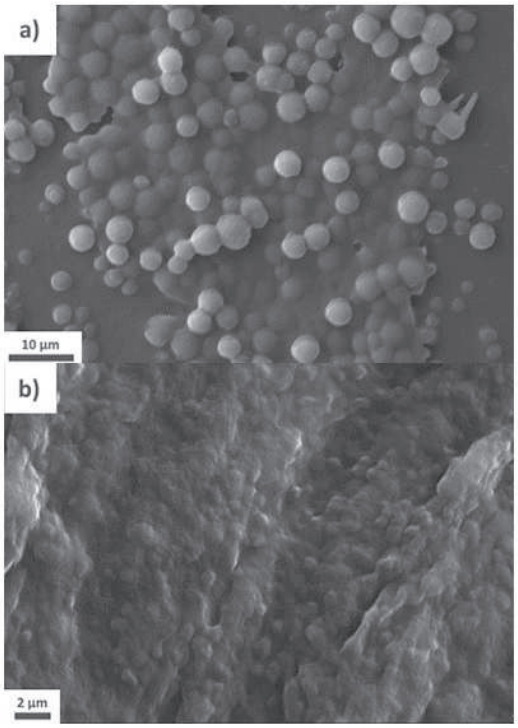

Figure 5. SEM images of xerogels formed from compound **3** (a), and compound **6** (b) in benzene.

Compounds **1** and **2** in benzene and compound **4** in toluene formed a fibrous gel network consisting of uniform-looking fibres (Figure 6). The width and length of the fibres varied within each system. The longest fibres were formed by compound **4** (from 61 μm to 775 μm) and the shortest fibres by compound **2** (from 12 μm to 242 μm). In the case of compound **1**, the length of the fibres varied from 19 μm to 376 μm. When comparing the width of the fibres, compound **4** possessed the thickest fibres (from 1.5 μm to 7.8 μm) and compounds **1** (from 654 nm to 6.6 μm) and **2** (from 732 nm to 4.7 μm) thinner ones. The texture of the gels formed in benzene and toluene was different. The gel formed by compound **4** in benzene was clearly fibrous (already to the naked eye), whereas the gel of **1** in benzene was cleavable. The gel formed by compound **2**, on the other hand, was clearly more ductile. It seems that the smaller and more even-sized fibres give rise to a more elastic gel as illustrated by compound **2** in benzene, and fibres with more versatility in size and shape produce less elastic gels as exemplified by compound **1** in benzene.

When inspecting the gel in acetonitrile and the partial gel in diethyl ether formed by compound **1** at a macroscopic level, both were fibrous. In the SEM images (Figures S1(a) and (b), respectively), both consisted of fibres in various sizes. Particularly, in acetonitrile, the gel network resembled a mixture of pieces of fettuccine and tagliatelle pasta. The length of the fibres varied from 24 μm to 244 μm, and the width from 1.5 μm to 6 μm. The average size of the plate-like structures amounted to approximately $37 \times 66\ \mu m^2$. In the partial gel formed in diethyl ether, the length of the fibres varied between 9.3 μm and 220 μm and the width from 863 nm to 13.5 μm, respectively.

The gels formed by compounds **1** and **4** in cyclohexene (Figures S1(c) and (d), respectively) and cumene (Figures S1(e) and (f), respectively) resembled each other. In the case of compound **4**, a correlation between the appearance of the gel and the structures seen in SEM images was observed. In both solvents, compound **4** formed a gel system consisting of

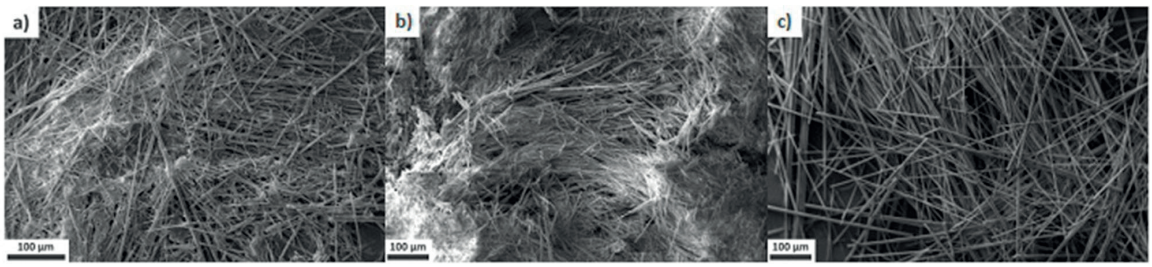

Figure 6. SEM images of compound **1** in benzene (a), compound **2** in benzene (b), and compound **4** in toluene (c).

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spherical shapes. In the SEM images, the gel network fibres were observed to form larger spherical structures. In cyclohexene, the length of the fibres was 27–314 μm and the width was measured to 309 nm–2.6 μm, whereas in cumene, the length of the fibres varied from 3.6 μm to 274 μm and the width ranged from 860 nm to 9.8 μm, respectively. The partial gel in cyclohexene formed by compound **1** was cleavable, whereas the partial gel in cumene consisted of spherical structures. Both of the gel systems were observed to consist of evenly distributed fibres with various sizes. In cyclohexene, the length of the fibres was 55–378 μm and the width was found at 1.4–13.6 μm. The partial gel formed in cumene by compound **1** resembled the gel formed in acetonitrile by the same compound, although in cumene, the fibres were smaller. The length of the fibres in cumene was 570 nm–14.8 μm and the width was measured to 7.9–239 μm, respectively.

The gel formed by compound **2** in ethylene glycol (Figure S1(g)), which formed extremely slowly, consisted of beam-like short fibres of various sizes. Their width varied from 1.7 μm to 6.3 μm, and their length ranged from 3.5 μm to 43 μm. The texture of the gel was notably more viscous and it stretched more than the other gel systems studied, an exception being compound **3** in benzene.

3. Conclusions

Compound **1** was clearly the most effective gelator molecule in the series of the six bile acid derivatives investigated within this study. Surprisingly, also a compound with deoxycholic acid backbone (**2**) formed gels. This has rarely been observed before. When comparing the number of the gel systems formed, the lithocholic acid-based compounds **1** and **4** formed the majority of the gel systems. The cholic acid derivative **3**, on the other hand, was the only compound, which exclusively formed flawless gels. This is speculated to be due to the ability of compound **3** to form hydrogen-bonded polymers, as was observed in the solid-state studies.

When comparing the compounds with respect to their side chains (linear hexyl side chains in compounds **1** and **3** and cyclohexyl side chains in compounds **4** and **6**), compounds with the linear hexyl side chain were clearly more effective gelators than compounds with the cyclohexyl side chain. Moreover, compound **2** with the deoxycholyl skeleton bearing the linear hexyl side chain formed gels, whereas the corresponding compound **5**, containing a cyclohexyl side chain, formed no gel systems.

The gel systems formed in this study were qualitatively more stable than gel systems produced in our previous studies.[33,34] They were, for example, stable against mechanical stimulus: when disturbed with a spatula, they maintained their shape or returned to a smooth-surfaced gel. In our previous studies,[33,34] the gel systems collapsed immediately after being mechanically disturbed. Previously studied bile acid alkyl amides bearing shorter or branched side chains (butylamides and *iso*-pentylamides, respectively) were observed to form a larger number of gel systems[33] than the currently studied **1**–**6**. Bile acid alkyl amides with even shorter side chains (ethylamides and propylamides)[33,34] were less effective gelators and the gels also decomposed easily. The effectiveness of gelation should thus be evaluated by taking into account not only the number of gels formed but also the qualitative stability of the gel.

The effect of an even larger branched or cyclic side chain or a longer alkyl group on the gelation process would be fascinating to study in the future, because the length and size of the side chain seems to have a major effect on the stability of the gel systems as observed in this study. These investigations could provide a step towards more accurate design of gelator molecules for desired purposes.

Experimental Section

Details of the syntheses, compound characterizations, and gelation studies are given in the Electronic Supporting Information. Deposition Number(s): 2085751 (for **1**), 2085752 (for **2**), 2085753 (for **3**), 2085754 (for **4**), 2085755 (for **5**) contains the supplementary crystallographic data for this paper. These data are provided free of charge by the joint Cambridge Crystallographic Data Centre and Fachinformationszentrum Karlsruhe Access Structures service.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: bile acid amides · intermolecular interactions · solvent influence · supramolecular gels · X-ray crystallography

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