Grafting Polyacrylates from Carbohydrates by ATRP and Formation of Nanoparticles for Advanced Drug Delivery

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Abstract

The present thesis describes synthesis and characterization of graft copolymers from carbohydrates and formulation of nanoparticles(NP)s for drug carrier application. Side chains were produced grafting from carbohydrate based macroinitiators via Atom Transfer Radical Polymerization (ATRP). Firstly, hydrophobic homo-, random- and block-copolymer side chains were grafted from a beta-Cyclodextrin core, followed by starch as a core. Monomer conversions up to 80% were achieved in most cases and well controlled polymers were obtained. Ni(II) triphenylphosphine complexes were found to be catalysts superior to the well known Cu(I) complexes, because they can be removed much easier from the product after polymerization.

NPs prepared by the emulsion-diffusion technique showed particle sizes of <200 nm, uniform shape and narrow size distribution (PDI<0.1). Not only the production parameter of NPs but also the chemical properties of the polymer influence the stability and size distribution of NPs. Especially the hydrophobicity of polymers and the composition of the arm of polymers (block or random) have a big influence on the stability and PDI of NPs. Since cell viabilities remain unaffected by these NPs, they are good candidates for the delivery of drugs. In conclusion, hydrophobically modified carbohydrate derivatives have a potential to serve as a new platform for biodegradable and biocompatible nano carriers.

Zusammenfassung

Die vorliegende Arbeit beschreibt die Synthese und Charakterisierung von Pfropf-Copolymeren aus Kohlenhydraten und Polyacrylaten, sowie die Formulierung von Nanopartikeln(NP) als Trägermaterialien für Arzneistoffe. Die Seitenketten wurden mittels Atom Transfer Radical Polymerization (ATRP) an die Kohlenhydrate anpolymerisiert. Zunächst erfolgte die Synthese von hydrophoben Pfropfpolymeren mit Homo-, Random- und Block-Copolymer-Seitenketten ausgehend von β-Cyclodextrin als Kern, anschließend wurde auch Stärke als Kern verwendet. Hierbei wurden unter Monomerumsätzen von mehr als 80% definierte Polymere erhalten. Ni(II)-Komplexe waren den bekannten Cu(I)-Katalysatoren überlegen, da sie nach der Polymerisation leichter entfernt werden können.

Die mittels Emulsions-Diffusions-Technik hergestellt NP, besitzen einheitliche Formen und Größen (ca. 200 nm) und eine enge Größenverteilung. Hierbei waren nicht nur die Parameter bei der NP-Herstellung, sondern auch die chemischen Eigenschaften des Polymers wichtig. Besonders die Hydrophobie der Polymere und die Zusammensetzung der Seitenketten (Block- oder Random) haben einen großen Einfluss auf die Stabilität und die Größenverteilung der NP. Da die Zellviabilität durch die NP nicht beeinträchtigt wird, eignen sie sich für pharmakologische Anwendungen. Die hydrophob modifizierten Kohlenhydrat-Derivate stellen somit eine viel versprechende neue Plattform für biologisch abbaubare und biokompatible Nanocarrier dar.

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Hiermit versichere ich eidesstattlich, dass ich die vorliegende Arbeit selbstständig und ohne Benutzung anderer als die angegebenen Hilfsmittel angefertigt habe.

Die aus anderen Quellen oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form in einem Verfahren zur Erlangung eines akademischen Grades vorgelegt.

Saarbrücken, Februar 2010 Masayuki Hirosue
Ludwigshafen, Februar 2010 Masayuki Hirosue

Abkürzungsverzeichnis

°C	Grad Celsius
AFM	Atomic Force Microscopy
AIBN	Azo-bis isobutyronitril
ATRP	Atom Transfer Radikal Polymerisation
BASF	Badische Anilin- und Soda-Fabrik
CD	Cyclodextrin
CuBr	Kupfer(I)bromide
DMF	N, N –Dimethylformamid
DMSO	Dimethylsulfoxid
DS	Substitutionsgrad, Degree of substitution
GPC	Gelpermeationschromatographie
HPLC	High performance liquid chromatography
IR	Infrarot (Spektroskopie)
M	Molar, Molmasse (g/mol), Präfix “Mega”
Min	Minuten
ml	Milliliter
MMA	Methylmethacrylat
mmol	Millimol
Mn	Zahlenmittel der Molmasse
MW	Gewichtsmittel der Molmasse
Ni	Nickel
NMP	N-Methylpyrrolidon
NMR	Nuclear Mass Resonance, Kernmagnetische Resonanz
NP	Nano Partikel
PDI	Partikel dispersitätsindex
pH	negativer dekatischer Logarithmus der Protonen-konzentration
PMMA	Polymethylmethacrylat
ppm	parts per million (NMR)
RAFT	Radikalische Addition Fragment Transfer
RT	Raumtemperatur
SFRP	stabile freie radikalische Polymerisation

Inhaltsverzeichnis

I. Einleitung

1.1.Nanopartikel als Carrier

Unter Nanopartikeln versteht man Teilchen, die eine Größe von 20 nm bis 1000 nm aufweisen. Die Größen der meisten beschriebenen Nanoteilchen liegen zwischen 100 und 1000nm. Im Gegensatz zu Makromolekülen besitzen Nanoteilchen eine eher kugelförmig kompakte Gestalt, während Makromoleküle im allgemeinen geknäult vorliegen und noch kleiner sind. Im Gegensatz zu Makropartikeln bilden Nanopartikel stabile kolloidale Lösungen, die auch parenteral verabreicht werden können, und die den Wirkstoff viel schneller freigeben. In den letzten Jahren sind nanoskalige Carriersysteme für Arzneistoffe zunehmend in den Fokus der Pharmazeutischen Forschung gerückt. Insbesondere Dank ihrer besonderen Fähigkeit, sich aufgrund höherer Permeabilität und niedrigerer lymphatischen Clearance im Tumorgewebe anzureichern, was man kurz unter dem Begriff „Enhanced Permeability and Retention Effect“ der kollidalen Systeme zusammenfasst. Allerdings litt die therapeutische Wirksamkeit der Systeme oft unter einer ungünstigen Anreicherung der partikulären Systeme(MPS), was zu einer ungünstigen Anreicherung der partikulären Systeme hauptsächlich in Leber, Milz, Lunge und Knochenmark führte. Dies konnte durch Modifikation der Partikeloberfläche mit PEG-Ketten erheblich verringert werden[1)].

Am günstigsten ist die „Core-shell“-Struktur der Nanopartikel, das Innere des Nanopartikels ist hydrophob und die Aussenschale ist hydrophil. Damit sind die Teilchen in Wasser gut dispergierbar und kann im Innern hydrophobe Wirkstoffmoleküle aufnehmen. Solche „Core-Shell"-Strukturen werden aus verzweigten amphiphilen Blockcopolymere hergestellt. Das Beladen eines solchen zweiphasigen Systems mit hydrophoben Wirkstoffen ist einfach durchführbar[2.3)].

1.2. Stärke und Cyclodextrin

Stärke und Cyclodextrin selbst sind schon lange bekannte natürliche Produkte in der Welt. Es sind jedoch auch sehr modische Forschungsgebiete, weil Stärke und Cyclodextrin sehr billig, essbar, umweltfreundlich und multifunktional sind[4.5)].

1.2.1. Stärke

Die Stärke ist da Nativ produkt und schon lange bekannt aus der Welt wie z.B. Reiskchen, Knudel. Die Stärke stellt neben Cellulose einer Hauptpolysaccharide dar und kommt in fast allen Pflanzen vor [6.7)].

1.2.2. Stärkemoleküle

Stärkemoleküle bestehen aus D-Glucose-Einheiten, die über glykosidische Bindungen miteinander verknüpft sind. Stärke besteht zu[8)].

20–30 % aus Amylose, dessen lineare Ketten eine helikaler (Schrauben-)Struktur

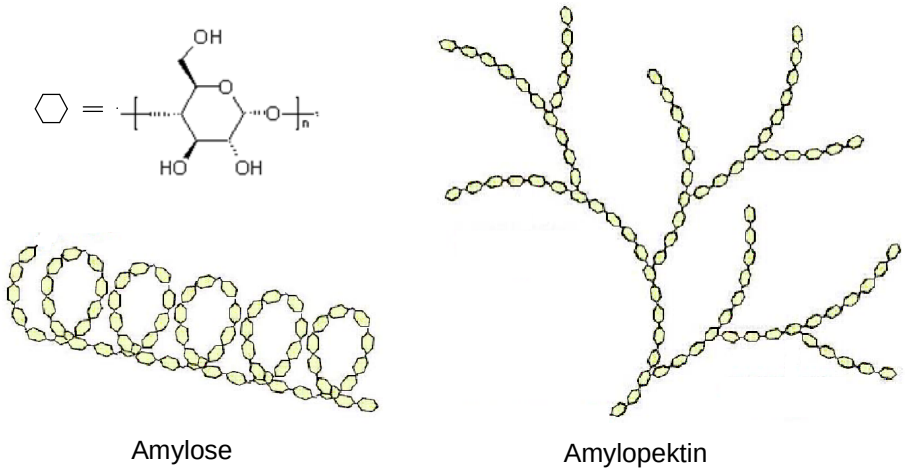


Abbildung 1. Die Struktur der Amylose und Amylopektin

aufweisen, in denen die Glucose-Einheiten ausschließlich α-1,4-glykosidisch verknüpft sind und 70–80 % aus Amylopektin, das eine stark verzweigte Struktur, mit α-1,6-glykosidischen und α-1,4-glykosidischen Verknüpfungen besitzt(Abbildung 1).

1.2.3. Enzymatischer Stärkeabbau [9-13)]

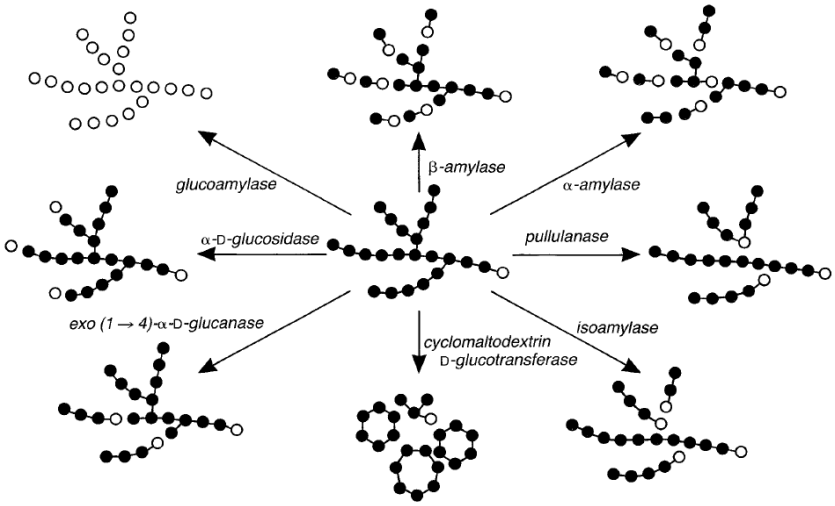


Abbildung 2. Enzymatischer Stärkeabbau

Durch Enzyme (α-, β-Amylasen) kann Stärke gespalten werden. Amylasen werden auch als Mehlbehandlungsmittel eingesetzt um Mehle besser backfähig zu machen. Speziell bei Roggen muss die Spaltung der Stärke in Folge natürlicher Amylase-Tätigkeit in der Regel jedoch eingedämmt werden, um die Struktur der Stärke zu steuern(Abbildung 2).

α- Amylase (EC 3.2.1.1) spaltet sehr effektive die α(1-4)-Glykosidbindung der Amylose. Dadurch entstehen Dextrine und daraus Maltose, Glucose und verzweigte Oligosaccharide(siehe Kapitel IV).

β-Amylase (EC 3.2.1.2) spaltet vom Kettenende her jeweils ein Maltosemolekül nach dem anderen ab. Diese Amylase kommt in Bakterien und Pflanzen vor.

Pullulanase (EC 3.2.1.41) ist bekannt als Pullulan-6-glucanohydrolase (Debranching enzyme). Dieses Enzym funktioniert nur in einer alpha-1,6-glycosidischen Verbindung(siehe Kapitel IV).

1.2.4. Anwendungsgebiete der Stärke

Die Anwendungsgebiete der Stärke sind sehr weitreichend. So wird sie

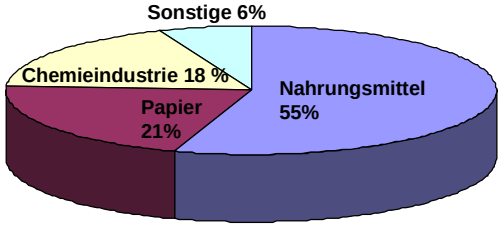


Abbildung 3. Stärkemarkt in die Industrie(BASF)

als Nahrungsmittel, bzw. in der Zubereitung selbiger, als Füllmaterial oder Binde- und Verdickungsmittel für Soßen eingesetzt. Aber auch für die Kunststoff-verarbeitende Industrie ist die Stärke von Interesse, da sie einen ständig nachwachsenden Rohstoff darstellt, der auch für die Herstellung von biologisch abbaubarem Einweggeschirr und andere umweltfreundliche Kunststoffe, wie auch Baumaterialien, geeignet ist. Darüber hinaus wird sie auch für haushaltsübliche Waren wie z.B. Waschmittel oder Stärke für Hemdkrägen verwendet. Außerdem stellt sie die Grundlage für die Herstellung von Hefe, Dextrin, Sorbit, Glucose (Traubenzucker) und Glucosesirup, aber auch für Kosmetik dar. Die Verkleisterungseigenschaften der Stärke und einiger Derivate macht man sich auch für die Herstellung von Tapetenkleister und Klebstoffe, aber auch für Schmierstoffe zu Nutze. Die guten Bindungseigenschaften kommen, wie beim Kochen, auch bei der Herstellung von Farben und Papier zum tragen. Selbst bei der Sprengstoffherstellung werden Stärkederivate, im speziellen Stärkenitrate, eingesetzt(Abbildung 3)[14-16)].

1.2.5. Anwendungsgebiete der Stärke in der Medizin

Auch in der Medizin sind Stärkederivate bereits auf dem Vormarsch. So wird derzeit an kaltwasserlöslichen Stärkederivaten zur Prophylaxe und Therapie von Blutvolumenmangel geforscht, wo sie auch schon erfolgreich als Plasmaexpander eingesetzt werden. Sie bilden außerdem die Grundlage für Tabletten, Zäpfchen oder Salben. Für die Verwendung im Bereich des Drug Delivery ist Stärke bereits für thermoplastische Materialien für Kapseln erforscht worden, ebenso, wie ihre Derivate als Kontrastmittel ins Auge gefasst werden[17-20)].

1.2.6. Cyclodextrin

Cyclodextrine (CD) gehört eine Klasse von Verbindungen die zu den zyklischen Oligosacchariden zu. Sie stellen ringförmige Abbauprodukte von Stärke her und bestehen aus α-1,4-glykosidisch verknüpften Glucosemolekülen(Siehe Abbildung 4). Deswegen entsteht eine toroidale Struktur mit einem zentralen Hohlraum wie Makkaroni Aussieht.

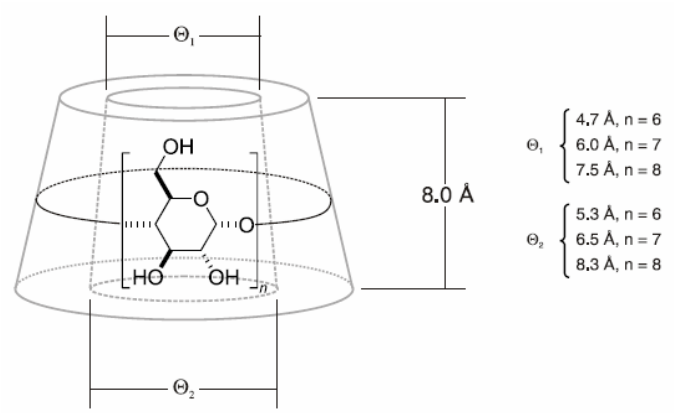


Abbildung 4. Cyclodextrin.

Cyclodextrine sind cyclische Oligosaccharide. Cyclodextrine haben über 6, 7 und 8 dieser Glucoseeinheiten und werden entsprechend mit α−, β− und γ− als Präfix gekennzeichnet. Die ist von enzymatisch aus Stärke, im industriellen hergestellten(Abbildung 4).

Diese und ihre verschiedenen Derivate, bei denen die freien Hydroxy-Gruppen durch diverse Reste ergänzt werden, wurden bereits erfolgreich auf Anwendungen wie Wirkstoff- oder Aromamolekül-Transport getestet und sind auch weiterhin ein markantes Forschungs- bzw. kommerzielles Produkt[21-24)].

1.2.7. Anwendung der Cyclodextrinderivate

Allen niederen Cyclodextrinen eigen sind die hydrophobe Kavität im Innern und die polare Außenfläche. Dadurch sind die Cyclodextrine in der Lage, so genannte Einschlussverbindungen mit apolaren organischen Verbindungen zu bilden. Diese Fähigkeit und ihre Wasserlöslichkeit machen sie zu einem immer wichtigeren Gegenstand der pharmazeutischen Forschung, da die Komplexe mit Pharmazeutika in der Regel besser wasserlöslich sind als die reinen Pharmazeutika und daher auch im Körper leichter verfügbar sind. Weiterhin ist ihre Fähigkeit, die eingeschlossene Substanz vor UV-Strahlung und umgebenden Verbindungen (z. B. Sauerstoff) zu schützen sowie die eingeschlossenen Substanzen über einen längeren Zeitraum abzugeben, von großem Interesse. In alkalischen Lösungen sind Cyclodextrine sehr stabil, in sauren Lösungen (bei einem pH-Wert kleiner als 3) werden sie dagegen zersetzt. Sie haben keine festen Schmelzpunkte, sind aber bis etwa 200 °C stabil.

Darüber beginnen sie sich zu zersetzen. Sie gelten als nicht toxisch und sind weitgehend stabil gegenüber menschlichen Verdauungsenzymen[25-30)].

Die in diesen Produkten befindlichen Cyclodextrinderivate binden die für unangenehme Gerüche verantwortlichen Verbindungen und sind gleichermaßen Träger von Duftstoffen. In manchen Ländern (z. B. USA, Japan) sind Cyclodextrine als Lebensmittelzusatzstoffe zugelassen, da sie unter anderem den Geschmack und Geruch der eingeschlossenen Substanzen aufheben oder wieder freigeben können. Das Einsatzspektrum von Cyclodextrinen umfasst heute von verschiedenen Medikamentenzubereitungen über Pappkartons bis hin zur Medizin, Landwirtschaft und Sensorik diverse Einsatzbereiche (Abbildung 5).

a)P&G b)Shiseido

Abbildung 5. Beispiel des kommerzielle Produkts mit Cyclodextrin.
a) P&G, b)Shiseido

1.3. Lebend Radikalische Polymerisation(LRP) [31-41)]

1.3.1. Freie radikalische Polymerisation(FRP)

Die Vorteile der radikalischen Polymerisation liegen in der Unempfindlichkeit gegenüber Verunreinigungen der Vielzahl kommerziell zugänglicher Monomere, deswegen ist diese Polymerisationstechnologie in der Industrie sehr wichtig (Abbildung 6).

Die freie radikalische Polymerisation(FRP) ist die am weitesten verbreitete Methode zur Darstellung einfacher, synthetischer Polymere. Sie ist eine Kettenreaktion, deren Ablauf im Wesentlichen durch drei Phasen, Kettenstart, Kettenwachstum und Kettenabbruch beschrieben werden kann. Der Kettenstart wird entweder durch Initiatoren mit Hilfe von Photonen oder thermischer Energie eingeleitet. Als Initiatoren werden z. B. Azo oder Peroxyverbindungen eingesetzt. Unter Wachstumsreaktion versteht man das Wachstum der molekularen Ketten in einer Kettenreaktion (Wiederholte Anlagerung der Monomere) bei der die makromolekulare Kette in einer Kettenreaktion wächst (wiederholte Anlagerung der Monomere).

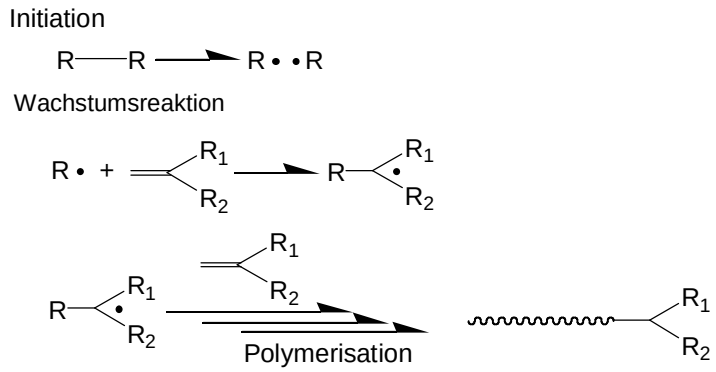


Abbildung 6. Initiaton und Wachstumsreaktion der FRP

Bei der Abbruchreaktion, wird das Wachstum der Kette durch Disproportionierungsreaktionen oder Kombinierung irreversibel beendet.

Zurzeit wird vor allem die freie radikalische Polymerisation in die Industrie verwendet. Abhängig von den Polymerisationsbedingungen, stellen wir fünf verschiedene freie Polymerisationstechniken vor.

1.Massenpolymerisation: Monomer als Lösungsmittel

2.Lösungspolymerisation: Monomer und Polymer in Lösungsmittel gelöst

3.Fällungspolymerisation: Monomer in Lösungsmittel gelöst, Polymer fällt aus

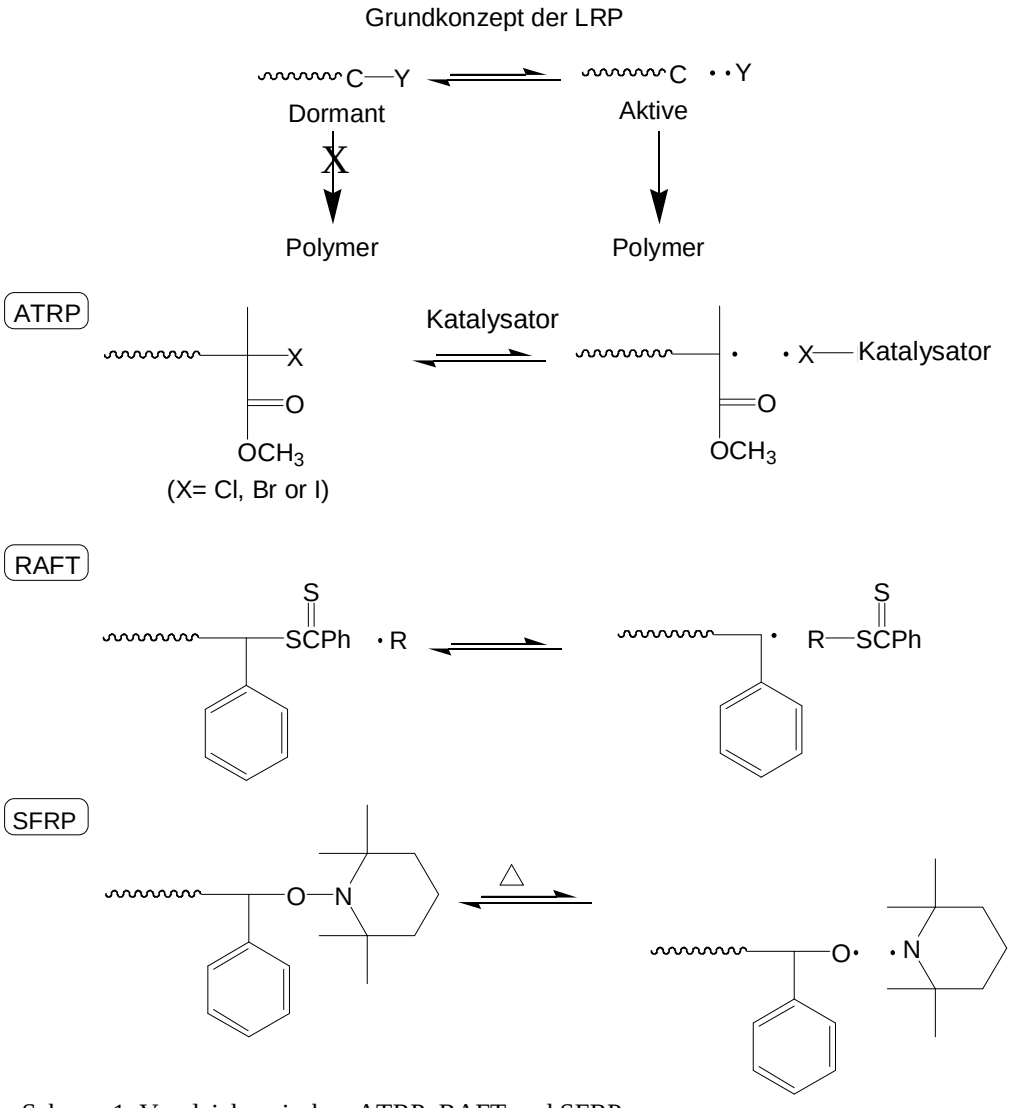
4.Emulsionspolymerisation: Monomer durch Emulgator in Wasser gelöst, Polymer fällt aus

5.Suspensionspolymerisation: Monomer durch Rühren und Stabilisatoren in Wasser suspendiert (kleine Tropfen), Polymer fällt aus

FRP ist wohl die optimale Methode Polymer herzustellen, allerdings hat sie den Nachteil, dass sich Blockpolymere nicht herstellen lassen, da sich das Molekulargewicht des Polymers nicht steuern lässt.

1.3.2. Lebend radikalische Polymerisation(LRP)

Mit Hilfe von LRP kann man sehr gut Molekulargewicht gesteuerte Polymere herstellen. Das ist sehr wichtig um Strukturgesteuerte Polymere herzustellen. Weil nicht alle freien radikalische Polymerisationtechniken geeignet für die Herstellung der Blockpolymere, Sternpolymere und Pfropfpolymere sind. Blockpolymere zeigen sehr spezielle Eigenschaften wie Micellen-Bindung, Schutz- Kolloide und Modifikation von Rheologie. Daher ist die Herstellung der Blockpolymere nicht nur im wissenschaftlichen Bereich, sondern auch in der Industrie von sehr großem Interesse. ATRP(Atom Transfer Radical Polymerization), RAFT(Reversible Addition-Fragmentation Chain Transfer) und SFRP(Stable free radical polymerization , diese beinhaltet auch NMP(nitroxide mediated polymerization)) sind 3 sehr bekannte Techniken der LRP(Schema1).

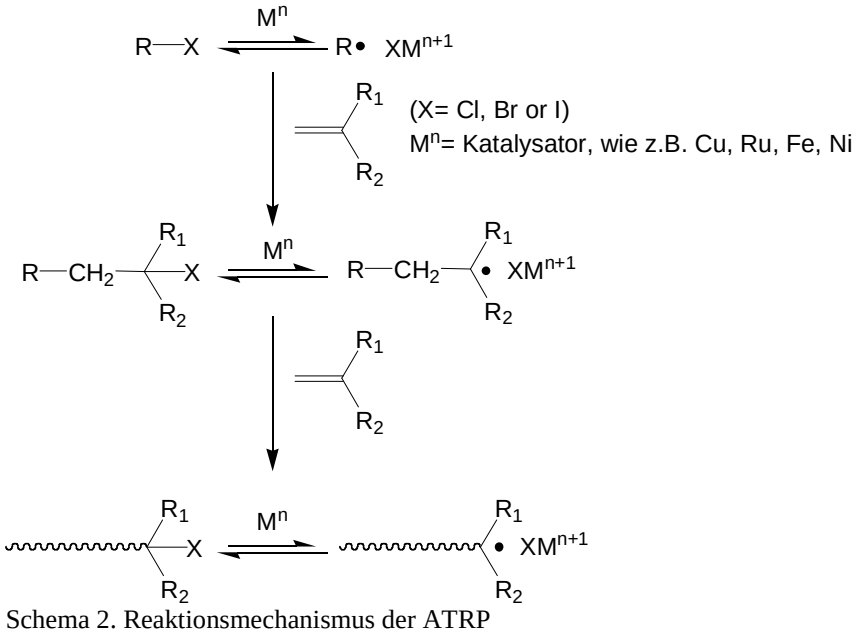


Schema 1. Vergleich zwischen ATRP, RAFT und SFRP

Alle Methoden haben Vor- und Nachteile. ATRP ist geeignet für viele unterschiedliche Monomere, aber die Entfernung des Cu-Katalysators ist immer schwierig. RAFT(Reversible Addition-Fragmentation Chain Transfer) ist eine günstigere Technik als die ATRP, aber man braucht bestimmte RAFT-Initiatoren oder Chaintransfer agents (CTA). Das Finden eines neuen universellen CTAs ist große Herausforderung in der RAFT-polymerisation. Bekannte und günstige CTA wurden schon patentiert. Allerdings sind die Lizenzkosten ein Problem, denn diese erhöhen die Produktionskosten enorm. Aus Kostengründen ist STRP die kommerzielle benutzbare LRP-technologie. Jedoch ist die Anwendung auf bestimmte Monomere begrenzt.

1.3.3. ATRP

Das Prinzip der lebenden Polymerisation wurde 1965 von Szwarc unter anionischen Bedingungen entwickelt[42)]. Die Entdeckung der lebenden Polymerisation bedeutete eine Revolution in der Polymer-Chemie. Durch die lebende Polymerisation lässt sich die Polymerstruktur kontrollieren. Pfropf-Polymere, Blockcopolymere und verzweigte Polymere können leichter produziert werden[43)]. Für eine lebende anionische Polymerisation sind jedoch nur wenige Monomere geeignet und es muss absolut wasserfrei gearbeitet werden. 30 Jahre später entwickelten Prof. Sawamoto (Japan) und Prof. Kennedy (USA) ein neues System der lebenden Polymerisation[44-45)]. Dies war die lebende kationische Polymerisation. Durch die lebende kationische Polymerisation wurde das Problem der begrenzten Monomerauswahl gelöst. Jeweils eine Firma in Japan (Kaneka) und in Deutschland (BASF) besitzt die notwendige Technik, um dieses Verfahren in der Produktion zu nutzen[46-47)] Trotz der größeren Auswahl an Monomeren besteht in diesem Punkt weiter Verbesserungsbedarf. Die bemerkenswertesten Fortschritte wurden 1995 von Prof. Sawamoto (Japan) und Prof. Matyjaszewski (USA) gemacht. Die Atom Transfer Radical Polymerization(ATRP) ist heute wegen des großen Interesses an strukturell klar definierten Polymeren ein vielbeachtetes Forschungsgebiet. Die Art des Katalysators ist der wichtigste Faktor für ATRP-Reaktionen [31-36,48-49)](Schema 2)



Schema 2. Reaktionsmechanismus der ATRP

1) Das Metallzentrum muss über mindestens zwei sich um ein Elektron unterscheidende Oxidationsstufen verfügen, deren Energiebarriere gering ist, sodass ein Wechsel zwischen beiden Oxidationsstufen leicht stattfinden kann.
2) Das Metallzentrum sollte eine entsprechend hohe Affinität zum Halogenatom aufweisen.
3) Die Koordinationssphäre des Metalls muss ungesättigt sein, damit eine oxidative Addition des Halogenatoms möglich ist.
4) Das Metallzentrum sollte vom verwendeten Liganden relativ stark komplexiert werden.

Die Erfüllung dieser Anforderung wurde bis dato für eine Reihe von Übergangsmetallen untersucht. Dabei wurde festgestellt, dass neben Cu auch Mo, Cr, Re, Ru, Fe, Rh, Ni und Pd Verwendung finden können [31-36)].

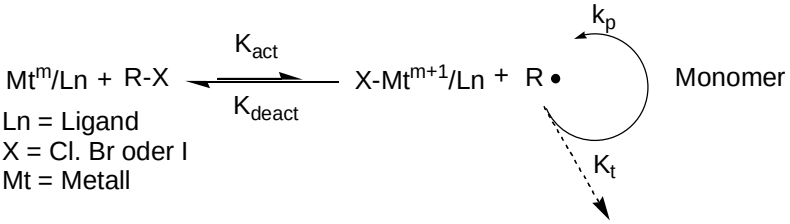
Verglichen mit RAFT oder SFRP hatte die ATRP bis 2001 den Nachteil, dass die gängigen Katalysatoren in Wasser unbrauchbar waren. Wegen der Stabilität der Katalysatoren läuft die Polymerisation in Wasser nicht gemäß dem ATRP-Mechanismus ab. In den folgenden 10 Jahren wurden jedoch wasserstabile Katalysatoren gefunden. Dadurch wurde die ATRP zur besten Technik zur Darstellung kontrollierter Polymere[31-36)].

ATRP ist geeignet für viele unterschiedliche Monomere. RAFT(Reversible Addition-Fragmentation Chain Transfer) ist eine billigere und günstigere Technik als ATRP, aber man braucht bestimmte RAFT-Initiatoren für bestimmte Monomere. Deshalb ist für die Herstellung von Blockpolymeren ATRP von Vorteil.

Trotz der Flexibilität in der Wahl der Monomere wird ATRP nicht oft in der Industrie verwendet. Ru-Katalysatoren können vielfältige Monomere umsetzen, werden aber aus Kostengründen nicht gerne genutzt. Cu-Katalysatoren werden nicht oft verwendet, da sie mit dem blauen $CuBr_2$ ein schlecht abzutrennendes Nebenprodukt ausbilden.

Ni katalysierte ATRP-Reaktionen wurden 1997 von Prof. Sawamoto (Japan) und Prof. Jerome (Belgien) entdeckt[50-54]. In den letzten 10 Jahren wurden jedoch keine Fortschritte erzielt, da die Katalysatorkosten zu hoch und der Anwendungsbereich zu begrenzt war. Weitere Untersuchungen im Bereich der Ni-katalysierten mit polarerem Phosphin ATRP-Reaktion bieten sich schon wegen der leichten Wasserlöslichkeit an. Dadurch sind diese Reaktionen besonders gut geeignet für Anwendungen im Bereich der Biochemie, Pharmazie und ähnlichen Bereichen mit dem Anspruch hoher Verträglichkeit und Reinheit und damit auch für das Nanostarch-Projekt.

1.3.4. Mechanismus der ATRP



Schema 3. Mechanismus der ATRP

In der Startreaktion bildet sich die aktive Spezies *R*• durch Transfer eines Halogenatoms zwischen einem Alkylhalogenid RX, das als Initiator fungiert, und einem Metall-Halogen(n)komplex von Cu, Fe oder Ru. Das aktive Radikal steht dann in niedrigen Konzentrationen für einen Wachstumsschritt zur Verfügung und wird nach erfolgter Addition einer Monomereinheit durch Reaktion mit dem entstandenen Metall-Halogen(n+1)Komplex wieder reversibel in ein Alkylhalogenid

(„dormant“ Spezies) überführt. Nachdem das Monomer aufgebraucht ist, kann die aktive Spezies durch Zusatz anderer Monomere zu Block copolymeren umgesetzt oder mit Hilfe geeigneter Abbruchsreagenzien terminiert werde[55-58)]
Die statistische Polymerisation verläuft unter Verwendung von Azo-bisisobutyronitrile(AIBN)-Initiatoren und nur über die „aktive“ Spezies, weswegen eine GPC-Untersuchung eine breitere Verteilung aufweist als bei einer ATRP-Reaktion. Die ATRP ist eine sogenannte lebende radikalische Polymerisation. Verschiedene Monomere können nacheinander polymerisiert werden und ergeben Block copolymere(Schema 3).

Die freie radikale Polymerisation mit Azoinitiator wie z.B. AIBN verläuft nur über die “aktive“ Spezies und führt deshalb zu einer breiten Molmassenverteilung wie GPC-Untersuchungen zeigen. Der Nachweis von Blockcopolymeren mittels GPC beweist, dass es sich bei der ATRP um eine kontrollierte („lebende“) Polymerisation handelt.

1.3.5. LRP und ATRP in der Industrie

Trotz des wissenschaftlichen Interesses an der ATRP, gibt es in kommerziellen Unternehmen nur „spezialisierte“ Anwendungsgebiete. Die Anwendungsmöglichkeiten der Polymere, die mittels LRP hergestellt werden, sind die Komponenten von Lackierung(coating, modifizierte Oberfläche), Klebstoffen, nichtionischen Tensiden, Dispergiermitteln, polaren thermoplastischen Elastomeren, Membranen, Produkten der Körperpflege, Oberflächenmodifikatoren, Hybriden mit natürlichen Polymeren und verschiedenen anorganischen, Bio- und elektronischen Materialien. Diese Anwendungsmöglichkeit basieren auf Patentanmeldungen zwischen 1995 und 2008[59)].

Mittels LRP hergestellte Klebstoffe werden in der Industrie wegen der hohen Herstellungskosten noch nicht eingesetzt.

Auf dem Elektronik- oder dem Pigment- Markt gibt es jedoch verschiedene Polymere, die mittels LRP hergestellt werden. Ein Beispiel der ersten Anwender von LRP war DuPont Performance Coatings, die heute einige kommerzielle Komponenten von Farben, Beschichtungen und Druckfarben mittels LRP herstellen. Die meisten

kommerziell wichtigen Polymerstrukturen sind Blockcopolymere, Di- und/oder Tri-Block-Strukturen. DuPont unternimmt sehr aktive Forschungs- und Entwicklungsanstrengungen in den allgemeinen Bereich der LRP. Das Unternehmen erwartet in den nächsten Jahren neue Produkte auf der Grundlage dieser synthetischen Techniken[60)].

Nicht nur Dupont, sondern auch Ciba Specialty Chemicals, Degussa, PPG, und Kaneka haben Polymere, mit Di-und/oder Tri-Block-Strukturen, mittels LRP, insbesondere der ATRP oder SFRP, produziert[61-63)].

Ciba konzentriert sich auf die Entwicklung der amphiphilen Pfropfcopolymere durch die Copolymerisation von Macromonomeren und anderen Monomeren mit Hilfe von ATRP und NMP, um genau definierte- / gesteuerte- Pfropfcopolymere herzustellen. Die Erste auf ATRP und NMP basierenden Produkte sind Blockcopolymere von Acrylicsäure, die unter dem Namen EFKA vertrieben werden.

RohMax Oil Additives, eine Tochtergesellschaft der Degussa, entwickelt wirtschaftlich interessante ATRP-Bedingungen um Zusatzstoffe von Schmierölen herzustellen. Diese Polymere sind Blockpolymere bestehend aus Poly (langen alkyl Ketten Methacrylat) und Poly (kurzen alkyl Ketten Methacrylat). Degussa hat auch das Know-how zur kommerziellen Herstellung von Blockcopolymeren und verfügt über die Technik den Katalysator vom Polymer zu entfernen.

PPG erwähnt einen weiteren wesentlichen Vorteil der ATRP, nämlich die Fähigkeit zur Manipulation. Dies erlaubt die Bildung von komplizierten Strukturen, wie zB Block-, Gradienten-, Kamm-, Stern- und Copolymeren. Sie verwenden diese als Bestandteile von verschiedenen Beschichtungsmaterialien.

Die Firma Kaneka entwickelt derzeit eine große Pilotanlage für die Telechelic-Materialien mittels ATRP. Aus Kostengründen ist die ATRP in der Industrie noch keine universell eingesetzte Methode. Das wird sich aber in den nächsten 10 Jahren deutlich ändern, da viele neue Produkte in absehbarer Zeit hinzukommen[64-66)].

1.3.6. RAFT

Wie beschrieben ist RAFT eine weitere Technik um kontrollierte Polymer herzustellen.

RAFT wurde erstmals 1998 von der Arbeitsgruppe um Rizzardo (CSIRO) entwickelt[67-71]. In erster Linie handelt es sich dabei um eine eigenständige Methode zur gezielten Synthese von Polymeren mit wohl definierter molarer Masse oder Polymerisationsgrad, geringer Polydispersität sowie bekannter Endfunktionalität.

Die Kontrolle der Reaktion wird durch reversible Kettenübertragungsreaktionen erreicht. Dabei addiert eine wachsende Radikalkette an die sogenannten RAFT-Agentien, wobei ein intermediäres Radikal entsteht. Auf Grund der Struktur der RAFT-Agentien hat dieses Zwischenprodukt die Möglichkeiten, zu verschiedenen Seiten hin zu zerfallen, wodurch entweder wiederum ein RAFT-Agents entstehen kann oder ein zur Kettenverlängerung zur Verfügung stehendes Radikal zurückgebildet wird, welches jedoch ausdrücklich nicht der originalen Radikalkette entsprechen muss.

Auf diese Weise wird die Propagations-Wahrscheinlichkeit über alle Ketten gleich verteilt. Die durchschnittliche Kettenlänge des gebildeten Polymers ist proportional zur Konzentration des RAFT-Agents sowie zum Reaktionsumsatz. Typische Substanzklassen für RAFT-Agentien sind Dithioester, Dithiocarbamate, Trithiocarbonate und Xanthate.

Ein Vorteil der RAFT ist die kontrollierte Polymerisation von Vinylmonomeren, wie z.B Vinylacetaten und Vinyl pyrrolidonen. die nicht durch ATRP erreicht werden kann. Die typischen Substanzklassen für RAFT-Agentien sind jedoch nicht kommerzielle Produkte und insbesondere im großen Maßstab schwierig in der Verarbeitung. Deswegen ist RAFT in Industrieprozessen noch nicht verwendbar.

1.3.7 SFRP (Stable free radical polymerization , das beinhaltet auch NMP)

Auch die durch stabile Reagentien, wie z.B. die durch die Nitroxid-Gruppe, vermittelte „lebende“ radikalische Polymerisation, beruht auf dem Prinzip des reversiblen Kettenabbruchs. Das wachsende Kettenende reagiert in einem reversiblen Gleichgewicht mit dem stabilen Reagens wie z.B Nitroxid-Radikalen, zu Alkoxyaminen und wird so in einen inaktiven, „schlafenden“ Zustand versetzt.

Ein Vorteil der SFRP ist die sehr kurze Reaktionszeit, dadurch ist sie für die Polymerisation von Styrol und Styrolderivat geeignet, die nicht einfach durch die ATRP polymerisiert werden können [37,72-76].

1.4. Sternpolymere und Pfropfpolymere mit Zucker

Stärkepfropfpolymere mit hydrophoben Seitenketten sind ein sehr kostengünstiges Produkt für thermoplastische oder kosmetische Anwendungen, und finden auch Einsatz in der Papier- und Biopharmazeutischen Industrie.
Die thermoplastischen Anwendungen als Ersatz für Erdöl-basierte Kunststoffe vor allem in der Verpackungsindustrie bilden einen weiteren Markt mit großem Potential. Aber aus Kostengründen und wegen der mechanischen Eigenschaften, befinden sie sich noch in der akademischen Phase.[77.78]

Um hydrophobe Stärkederivate herzustellen, wurden bereits 2 Herstellungsmethoden vorgestellt. (Graft-from Procedere und Graft-on Technik.)

Als Graft-on Technik, sind die Verätherungsreaktion und Verestherungsreaktion der Stärke im industriellen Maßstab bekannt. Zum Beispiel wurden Stärke-Acetate durch die Verestherungsreaktion mit Stärke und Essigsäure hergestellt [79].

Wegen des hohen DS von Stärke-Acetatal ist sie in unpolaren organischen Lösemitteln löslich. Ihre verzweigte Struktur macht sie jedoch schwerer handhabbar als Zellulose.

Deswegen haben die meisten kommerziellen Produkte lediglich einen niedrigen DS von 0,01 bis 0,2. Stärke-Acetate mit niedrigem DS werden sehr oft im Lebensmittelsektor verwendet. Bestimmte Lebensmittelanwendungen erfordern ein Stärkederivat mit einem milden Geschmack, Stabilität bei niedrigem pH-Wert und Stabilität in einem hohen Temperaturbereich. Überdies werden diese Stärke-Acetate in Säuglingsnahrung, Konserven, trockenen Lebensmitteln wie Instant-Saucen und Backwaren verwendet.

In den letzten Jahrzehnten hat sich die Pfropfpolymerisation von Monomeren wie z.B. Acrylaten oder Methacrylaten zu einer der universellsten und effektivsten Methoden entwickelt, um chemisch modifizierte, natürliche Polymere herzustellen.

Die chemische Modifizierung von Stärke über Vinyl-Pfropfkopolymerisation stellt eine wirksame Methode zur Verbesserung der Stärke-Eigenschaften dar, da Veresterungsreaktionen und Verätherungsreaktionen nicht als alternative Methoden in Betracht kommen.

Die Stärkepfropfpolymerisation wurde in der Vergangenheit mittels verschiedener Techniken durchgeführt, um Radikale in Hauptstärkeketten herzustellen. z.B. durch chemische Initiatoren wie Cer Ammoniumnitrat (CAN) [80-85], Kaliumpersulfat (KKS) [86] , Potassium Persulfat (PPS) unter Mikrowellenbestrahlung [87] und Ammoniumpersulfat (APS) durch reaktive Extrusion [88] , oder durch Bestrahlung ohne radikalen Initiator, wie Mikrowellen-Bestrahlung[89] und γ-Bestrahlung[90]. Diese Methoden für die freie radikalische Polymerisation von Vinylmonomeren sind schon sehr bekannt.

Sie sind vorzüglich geeignet, um Pfropfpolymere herzustellen. Allerdings kann die Länge der Seitenketten nicht so präzise gesteuert werden. Deswegen ist es schwierig die Eigenschaft von Stärke Polymeren zu reproduzieren.

1.4.1. Cyclodextrin Sternpolymer mit ATRP [91]

Seit 10 Jahren werden Sternpolymere im akademischen Bereich intensiv beforscht, weil Sternpolymere in den folgenden Anwendungen ein großes Potential haben:

- Wirkstoff Einschluß[92].
- Organische Reaktionen im Wasser [93-95].
- Erhöhen der Löslichkeit hydrophober Substanzen in Wasser [96-97].

Die Arbeitsgruppe von Haddlton verwendete 21 2-bromoisobutyl Ester als Initiator für eine ATRP Reaktion mit MMA und St und erzeugte damit Sternpolymere mit 21 Armen. Das war die erste Publikation von CD Sternpolymeren weltweit [98].

Müller stellte Sternpolymere mit Blockcopolymer Armen her, indem er n-Butylmethacrylat auf PMMA Sternpolymere polymerisierte. Diese Sternpolymere werden in der Thermoplastik angewendet [99-101)].

Die Nachfolgearbeit von Reynard war die Optimierung des Polymerisations Systems. Er erkannte, dass die Kombination von CuBr und Pentamethyl-diethylenetriamine (PMDETA) optimierte Bedingungen für die tert-Butylacrylat ATRP liefern[102)]. ATRP mittels Cu-Katalysators war exzellent für die Polymerisation der Monomere, aber die Entfernung des Katalysators ist immer noch schwierig, was insbesondere bei Pharmaapplikationen von Bedeutung ist. Stenzel hat einen alternativen Katalysator für die ATRP von St gefunden. Das ist die Halfmetallocene-Eisencarbonylkomplexe vermittelten ATRP. Titan (IV)-isopropoxid wurde auch als Co-katalysator verwendet[103)]. Unter Eisen-Katalysator Bedingungen erhält man Sternpolymere, die eine engere Molekulargewichtsverteilung haben vergleichbare Kupfer-Systeme. Allerdings ist die Farbe dieser Polymere wegen der Fe-Katalysatoren bräunlich.

1.4.2. Stärkepfropfpolymer mittels ATRP

Stärkepfropfpolymere mit Polyacrylate oder Polymethacrylat-Seitenketten wurden zuerst mittels Ce-Katalysator hergestellt. Aber mit dieser Methode kann man die Länge der Seitenketten nicht steuern. Diese Methoden leiden unter zu geringer Kontrolle der Polymerisation in Bezug auf Dichte und Länge der Seitenketten. Außerdem ist die Bildung von ungebundenem Homopolymer ein Problem. Die Überwindung dieser Probleme macht die ATRP von Acrylate und/ oder Methacrylate auf der Stärke zu einem derzeit sehr interessanten Forschungsgebiet.

Jedoch gibt es nach unserem Kenntnisstand bis jetzt nur 2 Berichte über Pfropfpolymerisation durch ATRP mit Stärke basierende Materialien [104)]. Polymerisation der nBA oder tBA wurde von Liu untersucht. Der erzielte Monomerumsatz erreichte dabei maximal 30% (Masayuki Ergebnis ist über 70%. Siehe Kapitel 3). Außerdem war die Verwendung von sehr hohen Mengen des Cu Katalysators notwendig und das Problem der Reinigung des Polymers vom Katalysator wird in diesen 2 Publikationen noch nicht gelöst[105)].

1.5. Zielsetzung

Das Ziel dieser Arbeit ist die Synthese von hydrophoben und/oder amphiphilen Stärke - oder Cyclodextrin Derivaten zur Ausbildung von Nanoteilchen und die Synthese der Vorstufen zu selbigen Derivaten(Abbildung 7).

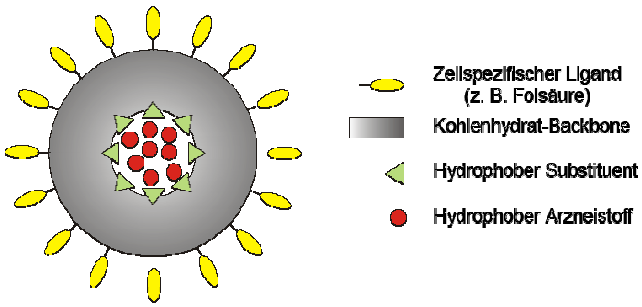


Abbildung 7. Konzept der Nanopartikel.

Hierbei soll in erster Linie noch kein Augenmerk der direkten Optimierung der Synthesebedingungen geschenkt werden, sondern hauptsächlich dem Auffinden eines generellen Syntheseweges von ATRP. In Zusammenarbeit mit dem Arbeitskreis von Prof. Dr. C. M. Lehr vom Institut für Biopharmazie und Pharmazeutische Technologie soll anschließend mit den hergestellten Derivaten eine Überprüfung der Anwendbarkeit im Hinblick auf die Herstellung von Nanopartikeln und einer Anwendung in der Medizin durch Einschluss von potentiellen Wirkstoffen erfolgen.

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II. Cyclodextrin-based Star polymer by Ni catalyzed ATRP: Synthesis and its application for nanoparticle formation

Abstract

The objective of this study was to synthesize hydrophobically modified cyclodextrins as a new platform for biocompatible and biodegradable nano-carriers. Star shaped polymers with a β-cyclodextrin (β-CD) core and hydrophobic arms composed of methyl methacrylate (MMA) and tert-butyl acrylate (tBA) were synthesized. The synthesis was realized by atom transfer radical polymerization (ATRP) using Nickel as a catalyst. The Ni^{2+} catalyst with a electron rich phosphine, such a tris(4-methoxyphenyl)phosphine made it possible to accelerate the polymerization. Using this system a monomer conversion of more than 80% was achieved under optimized reaction conditions. Moreover, the Ni catalysts are very easy to remove by simple washing with water. The obtained star polymers showed narrow molecular weight distributions. The solubility of the star polymer in ethyl acetate allowed the formation of nanoparticles (NPs) by the emulsion-diffusion technique. Resulting NPs were uniform and spherically shaped with particle sizes below 200 nm, and narrow poly dispersities (PDI<0.1). The MMA and tBA arms are expected to increase the encapsulation of active substances into the nanoparticles (NPs) and allow controlled drug release. These new CD-star polymers are a promising platform for preparing nanoparticle drug delivery systems since they do not show any cytotoxicity.

1. Introduction

Polymer nanoparticles are solid, colloidal particles in a size range between 10 and 200 nm consisting of e.g. inorganic materials or macromolecular substances. Polymer nanoparticles have attracted increasing attention over the past years for the delivery of active substances. They are applicable for advanced formulations of drugs, cosmetics, sun protection, health food, veterinary medicine or plant protection products. The development of nanoparticles for the controlled release of drugs, has improved the therapeutic methods in the recent years. Nanoparticles can improve the uptake and the plasma level of sparingly soluble pharmaceutical agents. Drug delivery systems for cancer therapeutics using polymer nanoparticles have revolutionized medicine. Delivery systems have improved the efficacy and reduced the toxicity of current therapies and resulted in the development of new ones. Targeted delivery systems of chemotherapeutics to the tumor compartment can be achieved systemically, either passively or actively. Nano-incapsulation radically changes the pharmacokinetics of the included drug, and nanoparticles target tumors passively via the enhanced permeability and retention (EPR) effect[1-5].

The development of nanoparticles as drug delivery systems for controlled release of drugs has improved the therapeutic methods. Besides, incorporation of agents in micelles, liposomes, nano-capsules or nanoparticles[6] were used to improve the bioavailability in particular of sparingly soluble actives. Although many different polymers were used to produce nano-carriers there is still room for improvement in terms of biodegradability and the achievement of well controlled polymerization conditions.

β-Cyclodextrin (β-CD) is a cyclic oligosaccharide consisting of seven glucose units linked by α-1,4-glucosidic bonds[7]. Due to the low toxicity of this natural sugar derivative, β-CD was used in therapy approaches and other biomedical applications, where it demonstrated promising results[8, 9]. In particular the 21 substitutable hydroxyl groups on the peripheral surface of β-CD provide the possibility to produce functionalized β-CDs, such as star shaped polymers[10, 11], CD based rotaxanes[12] and amphiphilic materials[13]. In this work β-CD was used as core for a controlled synthesis of star polymers with hydrophobic arm chains.

In order to get a well structure-controlled polymer product, atom transfer radical polymerization (ATRP), which is one of the controlled radical polymerizations (CRP), was used. CRPs are attractive alternatives to overcome most of the drawbacks of conventional free radical polymerization. Three methods for CRP namely RAFT (reversible addition-fragmentation chain transfer polymerization), SFRP (stable free radical mediated polymerization) and ATRP (atom transfer radical polymerization) were well reported in the last decade[14-17]. Especially ATRP is considered as a powerful technique to produce well structure-controlled polymers, such as graft polymer, block polymer, highly branched and star polymer because of its suitability to various kinds of monomers. Only star shaped polymer makes it possible to prepare highly concentrated micro core structure polymer or highly concentrated functional end polymers. Therefore star polymers are highly interesting polymers not only in academic but also in industry[18].

However, the major problem that limited the use of ATRP in industry in general and for pharmaceutical applications in particular, was the significant toxicity of the residual catalyst. In the field of cosmetic and medical products non-toxic polymers are inevitable. Nevertheless there is a demand in these application fields for star polymers because of their high functionality[14-17]. Many approaches have been conducted in order to reduce the residual amount of transition metal catalyst in the polymer product. For example, Matyjaszewski reported a new eco friendly green-ATRP concept[19] and also the use of an environmental friendly Fe catalyst ATRP system was puplished[20-24]. However, both of them still need an additional column process for the catalyst removal. A versatile applicable ATRP catalyst is of course of high interest for industrial use. Nevertheless the simple and effective product purification will be of impact for the product safety and the cost reduction of the synthesis/purification. Therefore a commercial ATRP catalyst which is easily removable from polymer by extraction into water is of high industrial demand[25].
Up to now only few ATRP systems were developed, which showed good polymerization kinetics and easy polymer purification using just water[20, 21]. In those studies it took a lot of effort to produce the catalysts with PEG unit or special P-N additives.

The use of Ni catalyst is also well known in ATRP[26-30]. For example Jerome produced a block polymer of MMA and tBA with $NiBr_2$ $(PPh_3)_2$ catalyst[30]. Muller and co-workers used $NiBr_2$ $(PPh_3)_2$ as an ATRP catalyst of glucose derivative monomers to produce a copolymer of sugar based acrylate and MMA[31]. Recently Sawamoto et al. reported that Ni catalyst with polar phosphine can be removed easily from polymer by water in polymer annual meeting Japan [17, 32, 33]. However, he used a Ni-salt as ATRP catalyst, which is very toxic and might be carcinogenic. In this study we examined a Ni based ATRP system without using this Ni salt. Instead $NiBr_2$ with tris(4-methoxyphenyl)phosphine as ligand was used as a safer alternative. Cyclodextrin-based star polymers were synthesized and checked for their qualification as nanoparticulate carrier system. A high level of safety is a prerequisite for biomedical applications. In this context natural polymer derivatives were used as material. Further the effective polymer purification from Ni catalyst using water extraction as well as the use of non-hazardous solvents during NP preparation was factors in order to maximize the biocompatibility of the produced NPs.

2. Experimental

2-1. Materials

β-Cyclodextrin (β-CD) (CAVAMAX®W7) was obtained from Wacker Chemie AG(Stuttgart, Germany) and dried in vacuum at 80°C overnight just before use. Methyl methacrylate (MMA) (Aldrich M55909: 99%) and tert-butyl acrylate (tBA) (Aldrich 327182; 98%) and dimethylaminoethyl metha acrylate (DMAEMA) (Aldrich 234907; 98%) were obtained from Sigma-Aldrich (Munich, Germany) or BASF (Ludwigshafen, Germany) and purified by passage through a column of activated basic alumina to remove inhibitor[34]. All other reagents were obtained from Sigma-Aldrich (Munich, Germany) and used without further purification. Polyvinyl alcohol (PVA) Mowiol 4-88 from Kuraray Specialties Europe GmbH (Frankfurt, Germany) and ethyl acetate from Fluka Chemie GmbH (Buchs, Switzerland) were used as obtained. Double distillated water was used to prepare nanoparticles.

2-2. Methods

Gel permeation chromatography (GPC) was performed as follows. Samples of the polymers were dissolved in the eluent (3 mg/mL), shaken for 16 h and injected by a 20 μL sample loop into a GPC system equipped with a refractive index detector Shodex RI-101 (Refractive Index Detector) with a flow rate of 1.0 mL /min. Data evaluation was performed by win GPC Unity Version 7.2 software from PSS (Polymer Standard Service.GmbH) Mainz, Germany. For the star polymers THF was used as the eluent with a Shodex PSS SDV 5 μm, 8.0*300 mm column (calibrated with narrow molecular weight poly styrene standards from PSS, Mainz, Germany).

UV spectra were taken with a UV/VIS spectrometer Lambda 2 (Perkin Elmer) in 0.1 or 0.5 weight % solution in THF or toluene. IR spectra were taken by a tensor 27 FTIR spectrometer (Brucker, Germany) from powdered samples with a golden gate diamond ATR unit.

Size and zeta-potential of the nanoparticles were analyzed by dynamic light scattering (DLS) and electrophoretic mobility, respectively, using a Nano-ZS (Malvern Instruments, Malvern, UK).

The morphology of particles was examined by atomic force microscopy using a Nanoscope IV Bio-scopeTM (Veeco Instruments, Santa Barbara, CA, USA). Imaging was done using taping mode and a silicon cantilever with a spring constant of approximately 40 N/m and a resonance frequency of about 170 kHz. The scan speed applied was 0.2 Hz.

2-3. Cyclodextrin based macroinitiator

β-CD (3.41 g, 3 mmol) was dissolved in 30 mL anhydrous 1-methyl-2-pyrrolidione (NMP) and was cooled to 0 °C for 2 h. α-Bromoisobutyryl bromide (29.0 mL, 126 mmol) dissolved in anhydrous NMP (50 mL) at room temperature was then added dropwise to the β-CD solution with magnetic stirring. The reaction temperature was maintained at 0 °C for 2 h and then raised slowly to 40 °C. Produced HBr gas during the reaction evaporated from one neck of the 3 neck flask. The reaction was continued for 6 h at 40°C and then overnight at room temperature. The product was washed first three times with water / THF and subsequently three times with water / CH_2Cl_2. The organic layer, which contained the product, was concentrated in vacuum. Drying over night in a vacuum oven lead to complete removal of residual solvent and yielded the macro initiator as a white powder. It was characterized according to Ohno and coauthors[10, 11].

2-4. General polymerization procedure

In general ATRP was preceded as following.

(a)

The polymerization was carried out under N_2 in baked 3 neck flask. $NiBr_2$ $(PPh_3)_2$ (149 mg 0.2 mmol), tris(4-methoxyphenyl)phosphine (140.1 mg 0.2 mmol) and DMSO (10 mL) were put into the reactor first. These solutions were stirred at 25°C overnight in order to allow the ligand exchange. MMA (2 g, 20 mmol) and macro initiator (0.04 g, 2 mmol initiating Br groups, 0.095mmol macroinitiator) was dropped into the reaction flask. The reaction mixture was stirred at 80°C for 4 – 24 h.

(b)

Monomer conversion was determined by the concentration of residual monomer measured by NMR using a certain amount 1.2-dichlorobenzene as an internal standard (^{1}HNMR signal at 7.2 ppm).

(c)

The polymerization was terminated by cooling the reaction mixture in an ice bath and exposure to air. First the polymer solution was diluted with reaction solvent and precipitated in water. Then the precipitate was filtrated off and dissolved in dichloromethane once again. It was extracted 3 times with 0.1 M HCl and with water. The organic phase was concentrated in vacuum. Polymer was dried over night in a vacuum oven at 65°C leading to a white powder.

(d)

Characterizations were as follows; the molecular weight and molecular-weight distributions were measured by SEC. The amount of residual catalyst was checked by UV/Vis spectroscopy (Absorption at 570 nm).

2-5. Preparation of nanoparticles

The star polymer was dissolved in ethyl acetate (1 mg/mL). This organic solution was dropped to 4 mL aqueous PVA solution (1% w/v). This biphasic system was emulsified with a high speed homogenizer (Ultra Turrax® Ika®, Brasil Ltda, Taquara, Brasil) at 14000 rpm for 15 minutes. Then, 5 mL ultra purified water (MilliQ water) was added to ensure diffusion of the organic solvent to the aqueous phase. Finally, the organic solvent was evaporated by stirring in an open vessel in the fume hood overnight at room temperature. The obtained nanoparticle dispersions were filled up to the end volume of 10 mL by addition of purified water. Characterization of the nanoparticle preparations was done by DLS and AFM as described above. Results are means of at least 3 measurements.

2-6. Cell culture

Caco-2 cell line clone C2BBe1 (ATCC No CRL-2102) passage no 70 was used as test system. DMEM No E-15-810 (PAA, Pasching, Austria) supplemented with 1% Non-essential amino acids (Gibco, Karlsruhe, Germany) and 10 % FBS (No P30-3300, PAN Biotech, Aidenbach, Germany) was used as growth medium. Subculture was done once a week at a subcultering ratio of 1:10 using trypsin-EDTA (No 25300, Gibco; Karlsruhe, Germany).

2-7. MTT assay

Thiazolyl Blue Tetrazolium Bromide (M5655, Sigma Aldrich) was dissolved in PBS pH 7.4 to yield a final concentration of 5 mg/ml for the stock solution.

Cells were seeded at a density of 10,000 cells per well in a 96-well cell culture plate. After five days propagation confluence was reached and MTT assay performed. Before exposure to particle suspension cells were washed twice with Krebs-Ringer buffer pH 7.4 (KRB). Particle suspension was prepared by centrifugation of the primary particle suspension to remove the water and resuspension in KRB supplemented with 10% FBS. Samples for the concentrations of 0.333 to 0.012 mg NP/ml were exposed to the cells.

After the incubation period of 3 h the particle suspension was removed. Cells were washed once with KRB before fresh medium with MTT reagent (0.5 mg/ml) was added. After further 4 h incubation 100 µl lysis buffer (10% SDS in 0.01mM HCl) were added to lyse the cells and solubilise the tetrazolium crystals. The absorbance at 550 nm was analysed in a plate reader. Viability was calculated in comparison to the negative control, untreated cells as 100% value, and positive control 1%-triton solution as zero% value. In parallel a cell-free assay with the same particle concentrations was performed to ensure that the colorimetric assay was not affected by the interactions with the nanoparticles

3. Results and Discussion

3-1. ATRP of MMA with Nickel catalyst from model initiator

The nickel complexes with 3 types of phosphine additives were used for polymerization of MMA started with the model compound, ethyl α-bromoisobutyrate[14-17, 19], in DMSO at 80 °C. A combination of bromide initiator and Ni catalysts induced smooth polymerizations without an induction phase, and conversion reached between 30 and 65 % in 24 h (Table 1)[35]. The effect of the type of phosphine ligand on the monomer conversion of MMA is shown in Table 1. Tris-(4-methoxyphenyl)-phosphine was the most efficient ligand resulting after 24 h reaction in a MMA conversion of 65 %.

Table 1. Effect of phosphine ligands on the ATRP polymerization of MMA.

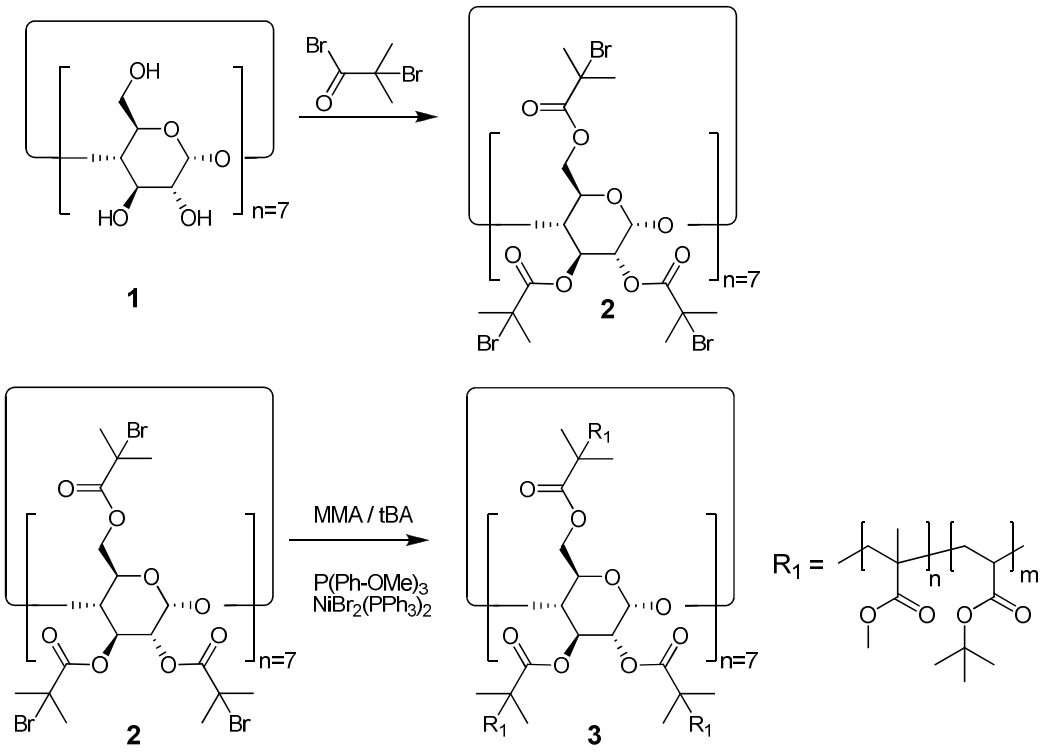
Entry [1)]	Type of Phosphine	pKa	MMA Conversion[2)] (%)
1	Tris-(4-methoxyphenyl)phosphine	4.59	65
2	Tri-p-tolylphosphine	3.84	35
3	Triphenylphosphine	2.73	30

1) MMA / initiator / $NiBr_2(PPh_3)_2$ / phosphine ligand = 4000 / 20 / 20 /40 mM in DMSO at 80°C
2) MMA conversion was measured after 24 h reaction time.

During ATRPolymerization, oxidation number of Ni atom changes between II to III. This electron transfer (reduction and oxidation system) makes it possible to control the concentration of radical species in the polymerization[13, 27-29]. We assumed that electron rich phosphine can stabilize the Ni(III) catalyst during the polymerization, which is a 17e low stability complex compared to the 16e $NiBr_2(PPh_3)_2$. This gives rise to the high speed and well controlled ATRP. Ouchi and his coworkers also reported that the same phenomena, of phosphine ligands influencing the polymerization kinetics of MMA in ATRP, observed in the case of Ruthenium catalyzed ATRP[36]**.** In this study we learn that the basicity of phosphine influenced the MMA ATRP polymerization rate. Furthermore, since tris(4-methoxyphenyl) phosphine is soluble in acidic water, the purification process of the polymer is very simple. Usually the removal of the ATRP catalyst from obtained polymer was a great challenge[24, 25].

3-2. Synthesis of cyclodextrin star polymers with MMA arms

Reaction scheme for the synthesis of a star polymer is introduced in scheme 1. Step 1 is to produce the macroinitiator **2**. Step 2 is to synthesize the star polymer **3**. To prepare the star polymers via ATRP from the CD cores, it is necessary to introduce alkyl halide initiators onto the CDs (Scheme 1, step 1). It has been reported that ATRP is carried out from a multifunctional core with a high local concentration of initiation sites, radical-radical coupling of the propagating chains will probably occur and result in gelation[10, 11].



Scheme 1. Synthesis of cyclodextrin star polymers by ATRP

In order to inhibit possible gelations, we examined polymerization at low monomer concentrations of 400 mM. The synthesized β-CD derivative **2** was used as an initiator for the Ni(II)-mediated living radical polymerization of MMA and tBA to produce 21-arm star polymers using $NiBr_2L_2$ (L = tris(4-methoxyphenyl)phosphine) as the catalyst (Scheme 1). Several polymerization parameters, including reaction temperature, reagent concentrations, and overall concentration, were varied to optimize the conditions to prepare well-defined star polymers. Table 2 summarizes the results of these polymerizations.

Table 2. Synthesis of CD star polymers with PMMA arms

Entry[1)]	Monomer/ Initiator	Central metal	Conversion (%)	M_w	M_w/M_n
4	20	Ni	94	3.1 10^4	1.51
5	50	Ni	97	1.0 10^5	1.58
6	50	Fe	94	1.4 10^5	1.92

1) MMA / macroinitiator / catalyst / tris-(4-methoxyphenyl)phosphine = 400 or 1000/20/ 20 / 40 mM in DMSO or toluene at 80 °C

Initially, the HOMO polymerization of MMA was carried out with a starting concentration ratio of monomer to inititor $[M]_0/[I]_0$ of 20 at 80 °C in toluene (entry 4), where $[I]_0$ is the initial concentration of initiation sites in compound **2**. Molecular weight of obtained polymer was measured by GPC calibrated with polystyrene standards**.** Molecular weight distribution was well controlled (M_w / M_n= 1.51) and no shoulder peak was observed in the higher or lower molar mass region in main peak. This confirms that ATRP runs without any side reaction and star polymer was synthesized. Appearance of a shoulder at the main peak in the higher molar mass region would be an indication of irreversible coupling of two star polymers[10]. Star-Star coupling is mostly observed at the end stage of polymerization, because of its high molecular weight of star polymer and highly concentrated reaction conditions.

In case of $[M]_0/[I]_0$ ratio of 50, obtained polymer showed relatively controlled distribution (M_w / M_n= 1.58). Fe catalyst was also used in ATRP because of its low cost and low toxicity compared to the other ATRP catalysts[20-22]**.** Therefore PMMA star polymer with β–CD core was also synthesized by Fe-catalyst. $FeBr_2$ complexed with tris(4-methoxyphenyl)-phosphine was reacted with MMA and macroinitiator **2** in DMSO at 80 °C under homogenous conditions. MMA was consumed more than 90% within 24 h but molecular weight distribution of the obtained polymer (M_w / M_n) was relatively broad 1.92. Additionally, even after washing several times with water, rusty red color still remained on the polymer and it was impossible to remove the iron completely from the polymer (residual amount 92 ppm). For the desired nano-medical applications, the purity of polymer is one of the most important factors. Hence, best polymerization parameters were the following: reaction temperature at 80 °C, reagent concentrations of 20 mM Ni catalyst and 40 mM phosphine.

In conclusion, Ni catalyst was superior for nano-medicine applications compared to Fe catalyst, because Ni catalyst made it possible to achieve the narrower polymer distribution.

3-3. Synthesis of star polymers with copolymer arm

The random copolymerization of MMA and tBA was carried out with an $[M]_0/[I]_0$ ratio of 20 at 80 °C in DMSO (Entry 7). Figure 1 shows the monomer conversion of MMA and tBA in the random copolymerization. MMA and tBA was consumed 1st linear plot against reaction time. This result indicated that polymerization occurs in controlled polymerization process. After 24 h, a monomer conversion of 96 % (MMA) and 89 % (tBA) was achieved. The resulting polymer was washed with water three times, to obtain a white and odorless powder. UV spectrum of the finally obtained star polymer showed no peak around 570 nm (Ni catalyst d-d transfer), which indicates that almost all Ni catalyst was removed from the polymer after washing 3 times with water (<30 ppm Ni as obtained by combustion analysis).

Table 3. Synthesis of CD star polymers with poly(MMA-co-tBA) arms

Entry[1)]	**Monomer/ initiator (Br)**	**Solvent**	**Temperature / °C**	M_w **(GPC)**	M_w/M_n [3)]
7	20	DMSO	80	$3.0\ 10^4$	1.54
8[2)]	20	DMSO	80	$2.2\ 10^4$	1.74
9	50	DMSO	80	$1.7\ 10^5$	2.36
10	100	DMSO	80	$2.1\ 10^5$	2.02

1) MMA / tBA=1/1, monomer / macroinitiator / catalyst / tris-(4-methoxyphenyl)-phosphine = 400 , 1000 or 2000 /20/ 20 / 40 mM, in random co-polymerization
2) Block copolymer
3) Polymerization was stopped after 24 h reaction time. Monomer conversion was over 70%

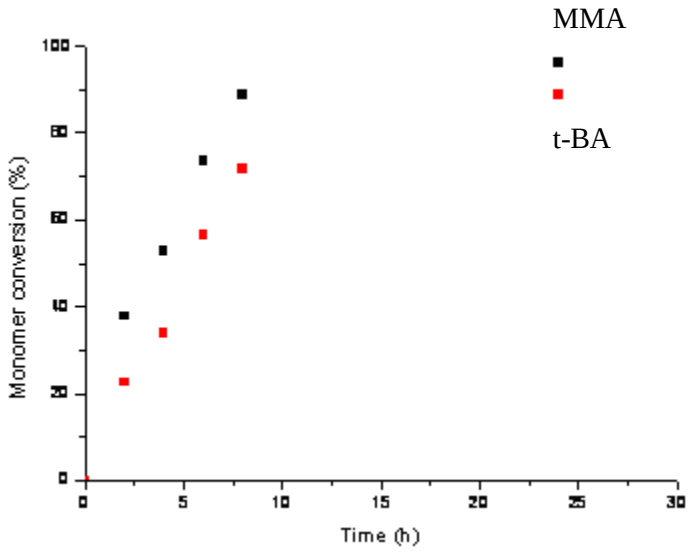


Figure 1. ATRP polymerization kinetics of MMA / tBA from CD derivative **2**

Secondly, the block copolymerization of MMA and tBA was carried out with an $[M]_0/[I]_0$ ratio of 10 MMA units and 10 tBA units at 80 °C in DMSO (Entry 8). After 70% MMA monomer was consumed, tBA was feed into the glass flask. Star polymer with block copolymer of MMA and tBA as a side chain was produced. Obtained polymer showed relatively broad but acceptable as a block polymer distributions (M_w/M_n=1.76). No shoulder peak in higher molecular weight was observed at the end of polymerization. This indicates that star-star coupling was not preceded even after the block polymerization. The further study in regarding to block polymer is in progress in our group now.

In order to obtain a long arm length star polymer, $[M]_0/[I]_0$ ratio was changed from 20 (Entry 7) to 50 (Entry 9) and 100 (Entry 10). Polymerization was preceded at 80 °C in DMSO like Entry 7. After the 24 h polymerization, Entry 7 achieved 91 % monomer conversion. Run 9 achieved 81% monomer conversions. In case of Entry 10, due to its too high viscosity, monomer conversion was achieved only 70 %. Therefore we learn that the length of the side chain and viscosity of reaction mixture affects the polymerization speed. The longer the side chains the slower the polymerization speed. We assume that star-star coupling in the long arm star polymer makes the polymerization slower and star-star coupling form a highly cross linked polymer and cause a high viscosity. This hypothesis was backed by the GPC results of entry 10 polymer, which revealed a shoulder in high molecular weight. The radical species

were consumed during the coupling reactions. Hence the polymerization could not run any more. In other words, star-star couplings inhibit the further monomer consumption.

The advantage of the synthesis of MMA-tBA copolymers as the side chains is to create CD star polymers with amphiphilic, or zwitter ionic arm easily. These further studies to produce star polymers with amphiphilc arms are now in progress.

3-4. Optimization of the synthesis of the starpolymer 3

The potential to reduce production costs is a key to success in industry. Therefore we examined ATRP in other solvents other than DMSO to achieve a lower boiling point to reduce energy consumption necessary for the isolation of the product. Random copolymerization of MMA and tBA was carried out with the same Ni catalyst at 80 °C in toluene, 60°C in THF and 80°C in acetonitrile. Monomer conversions of MMA of 83% (in toluene), 16% (in THF) and 48% (in acetonitrile) were achieved.

This result indicates that reaction solvent influence the catalyst activity and monomer conversion. The charge transfer between d-orbital and d-orbital (d-d transfer) of Ni catalysts in toluene, THF and acetonitrile was observed by UV spectroscopy. In case of THF and toluene, d-d orbital peaks of Ni catalysts were observed at 580 nm. In contrast in case of acetonitrile, d-d peak was detected around 600 nm. This differences show that solvents, which have a strong electron donating part like the cyano group, influence the central metal ion of the catalyst. We assume that strongly coordinated cyano group will change the redox equilibrium between Ni(II) and Ni(III) during the reaction, so the polymerization becomes slower.

In case of polymerization in toluene, GPC curve of obtained polymer was very sharp without any shoulder. (M_w/M_n =1.13). The obtained polymer was well controlled compared to the DMSO (entry 7). Depending on the solvent, color of catalyst solution was changed during the reaction. These finding also indicates that the oxygen atom of DMSO influenced the central Ni catalyst. Then we assume that the influence of the central Ni metal changes the polymerization kinetics. Hence even though the polymerization runs faster in DMSO than in toluene, the polymer distribution index (M_w/M_n) in DMSO becomes broader than in the polymerization in toluene. From the

point of view of polymerization optimization toluene would be superior to DMSO. However, in order to apply our polymer in medical application, the low toxicity is the highest priority. Therefore we decided to choose DMSO as solvent, because DMSO is less toxic solvent than toluene (See MSDS data sheet, DMSO is no R-figure but toluene has R11, R38, R48/20, R63, R65, R67) and still yields in a good polymerization speed and fairly narrow polymer distribution.

3-5. Colloidal characterization of the NPs

Heptakis-(2,3-di-O-acety)-β-CD (CD-ref) was synthesized as a low molecular weight reference compound according to a procedure published by Chankvetadze[37]. Because natural β-CD was not enough soluble in ethylacetate, CD-Ref was synthesized. The hydrophilic nature of β-CD limited their application in nanotechnology. Trials to prepare NPs with CD-Ref were not satisfactory. The mean particle size was 185.5 nm, but the high polydispersity index of 0.38 indicated a broad particle size distribution. The limited hydrophobicity of the CD-Ref may be the reason for the low functionality in nanoparticle formation. The addition of MMA-tBA arms to β-CD resulted in the formation of star polymers that are hydrophobic enough to form nanoparticles (Figure 2, polymers were chosen from entry 7-10). The obtained particles were in the size range between 170-185 nm with a uniform, narrow size distribution (PDI = 0.06 – 0.13). The colloidal properties of the particles were maintained by using PVA, which sterically stabilizes the dispersion. Measured zeta potentials in aqueous dispersion were approximately -3 mV. Without addition of the emulsifier PVA no stable nanoparticle dispersion could be achieved. A typical size-distribution obtained by dynamic light scattering (Fig. 3 a) and a typical AFM image (Fig. 3b) of obtained nanoparticle based on synthesized star polymer (entry 7 in Table 3) is shown in Figure 3. The AFM picture illustrates the spherical shape of the NPs and their smooth surface. Consequently, these β-CD star polymers represented are suitable for the formation of nanoparticulate drug carriers. Investigations about the encapsulation of active substances are now in progress.

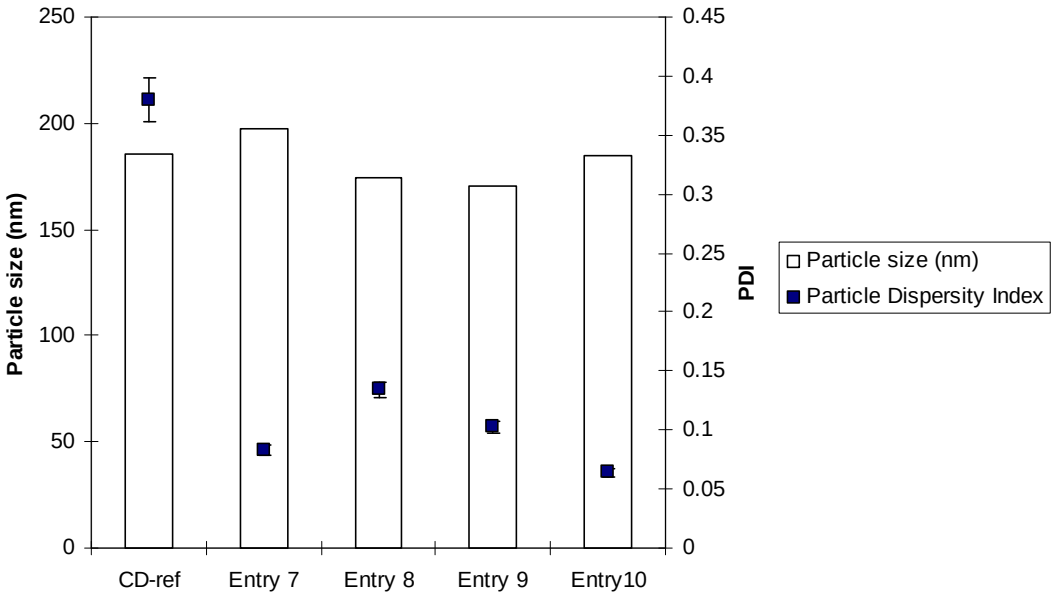
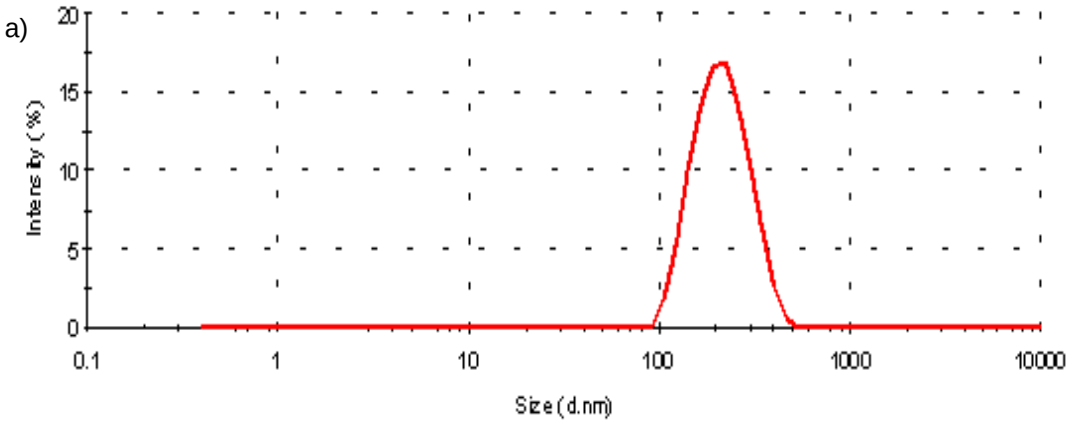


Figure 2. Characterization of CD-star polymer nanoparticles. Square point shows the polydispersity indes (PDI) and bar shows the mean particle size (nm) determined by DLS.



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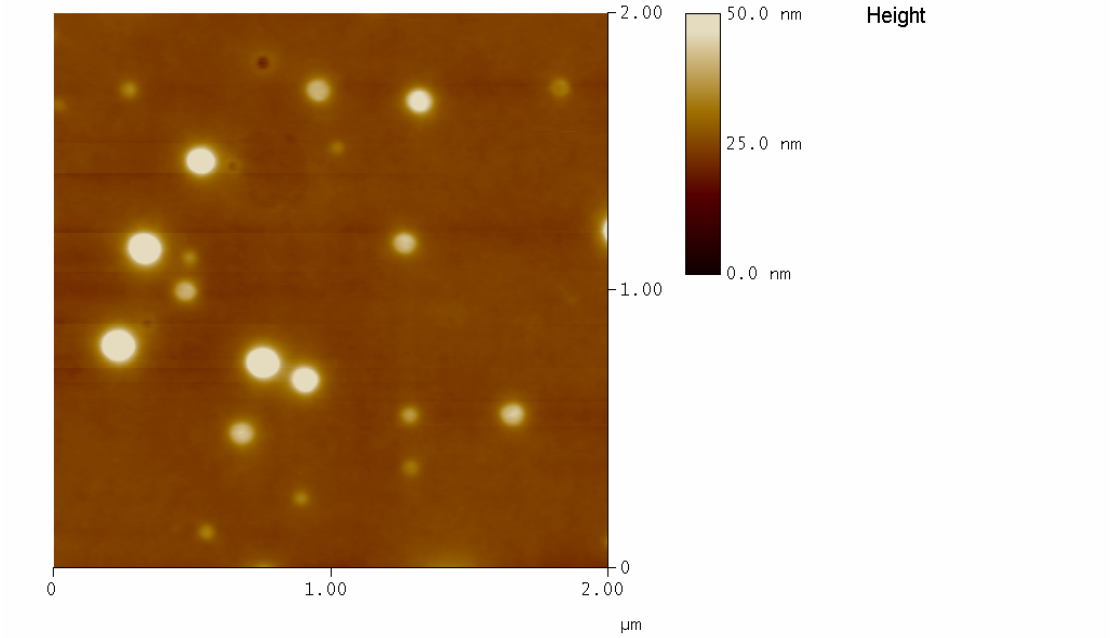

c)

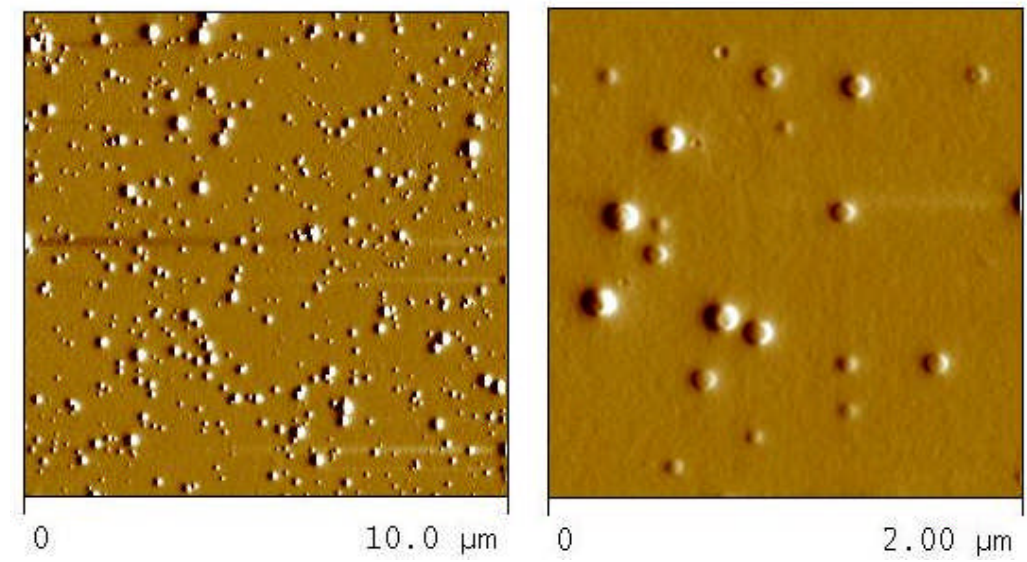

Figure 3. Colloidal characterization of β CD-Star polymer with MMA and tBA arm chains NPs (Polymer, entry 7 was used)

a) NPs intensity weighted size distribution measured using DLS
b) Morphology of NPs monitored by AFM using Height mode
c) Morphology of NPs monitored by AFM using Amplitude mode

3-6. Stability of nano particles.

Three different β-CD star polymers with MMA and tBA arm chains or MMA homo polymer side chains were used to prepare NPs for the stability test. Nanoparticle preparations were stored at 4°C in the refrigerator for 6 weeks. Particle size was analyzed immediately after preparation and subsequently after 3 and 6 week of

storage. Table 4 summarizes the results. All three nanoparticle preparations kept their initial size and size distributions for 6 weeks.

Table 4. Stability of nanoparticless of 3 β-CD star polymers.(Mean ± SD, n=3)

Entry	Side chain	Length	M_w/M_n	Nanoparticle		After 3 weeks		After 6 weeks	
				Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI
1	MMA-tBA copolymer[1)]	20	1.6	190.1 ± 0.075	0.077 ± 0.023	191.5 ± 2.16	0.084 ± 0.033	188.2 ± 0.2	0.078 ± 0.007
2	MMA-tBA copolymer[1)]	20	1.06	177 ± 1.51	0.059 ± 0.005	181.2 ± 0.071	0.064 ± 0.064	179.5 ± 2.26	0.062 ± 0.013
3	MMA-homo polymer	50	1.58	184.8 ± 2.01	0.086 ± 0.022	189.2 ± 0.9	0.049 ± 0.049	184.4 ± 2.37	0.075 ± 0.018

1) MMA/tBA=1/1

3-7. Nanoparticle study; toxicity

The obtained star polymers were hydrophobic enough to offer a promising platform for preparing nanoparticulate carrier systems. The possibility to remove the catalyst (star polymer entry 7, residual Ni content 30 ppm) and the use of non-hazardous solvents make these NPs a promising candidate for drug delivery systems. Therefore to prove the toxicity of NP with star polymer, cytotoxicity was analysed using MTT assay, which monitors the mitochondrial metabolism of cells as an indicator of their viability.

Figure 4 displays the MTT results after a 3 h incubation period with nanoparticles based on the star polymer (sample entry 7 in Table 3). The tests demonstrated that concentrations up to 0.333 mg NP/mL are not cytotoxic in the Caco-2 test model. A deviation of some percent in viability is considered normal in cell culture. The standard deviation of the untreated cell controls is indicated as dashed lines. The observed results for all tested concentrations are within the standard deviation (Figure 4).

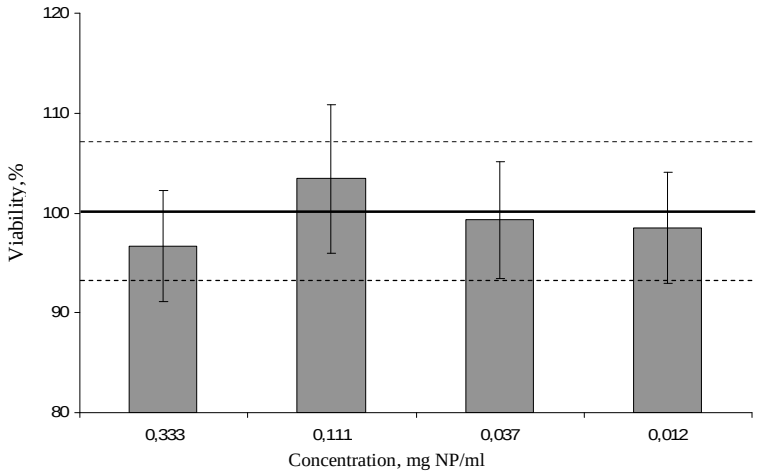

Figure 4. Cytotoxicity test of CD star polymer particles (entry 7 in Table 3) for a Caco-2 cell culture.(Mean ± SD, n>3).

4. Conclusion

Star shape polymer with a β-CD core and hydrophobic arms were produced by ATRP using Nickel catalyst. Polymerization of MMA and tBA were preceded and more than 80% monomer consumption within 24 h could be achieved in an optimized system. Ni catalyst with a hydrophilic phosphine such a tris-(4-methoxytriphenyl)phosphine allows to accelerate the polymerization speed and facilitate the polymer purification, which can be performed by water extraction. Furthermore we investigated the impact of solvent on the polymerization kinetics and product.

The obtained novel star polymers were hydrophobic enough to offer a promising platform for preparing nanoparticulate carrier systems. The possibility to remove the catalyst (below 30 ppm) and the use of non-hazardous solvents make these nanoparticles a promising candidate for drug delivery systems.

5. Acknowledgement

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III. Optimization of process to produce Cyclodextrin based star polymer and evaluation as material for nanoparticle preparation

Abstract

Methyl methacrylate(MMA)-tert-butylacrylate(t-BA) blockcopolymer arm chain star polymer (hydrophobe-type)and MMA and acrylic acid (AA) copolymer arm chain (amphyphilic type) star polymers, whose composition and length of the arm chain were tuned, were produced by Nickel catalyzed atom transfer radical polymerization(ATRP).

By using such a controlled size star polymer (M_w/M_n<1.3), strategies to produce Nanoparticles were investigated by the authors and introduced in this publication. We clarified that not only the production parameters such as stirring speed and concentration of emulsifier but also chemical effects for example molecular weight of arm chain and composition of star polymer influence the stability and particle size distribution of NPs. Especially hydrophobicity of star polymer and the composition of the arm of star polymers (block or random) had a big impact on the stability and particle size distribution of NP. However molecular weight of star polymer did not have a significant impact on the stability of NPs.

1. Introduction

NEW DRUGS such an idarubcin has a high potential for cancer therapy but it is not water soluble. Therefore nanoparticles (NP), which encapsulates hydrophobic drugs with polymer, provide a good method to improve their solubility. Thousands of polymers were produced as polymers for drug delivery systems to our study, however NP with native polymer are still a very active research area up to now due to their favorable toxicological properties like biocompatibility and biodegradability[1-3].

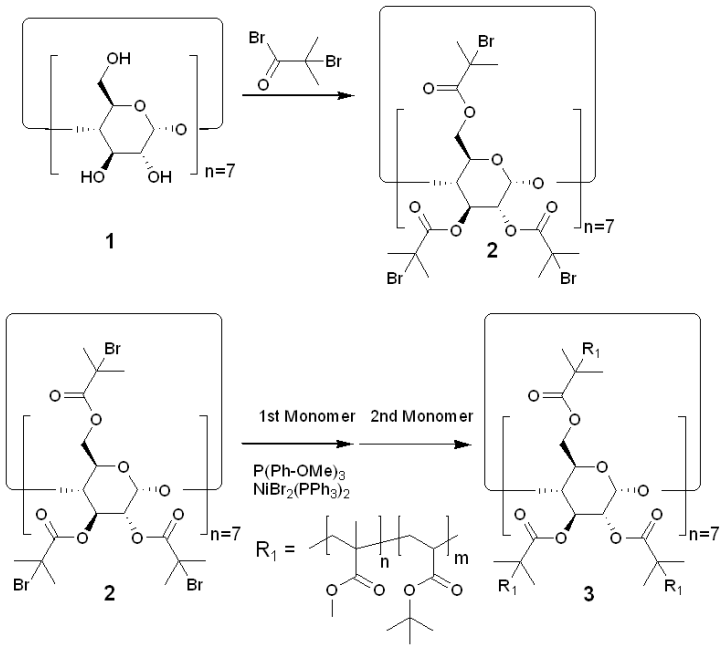
NP with starch-based polymers to improve the solubility of drugs have often been published in this decade. Ortega, M. and co-workers produced a propyl-modified starch to encapsulate hydrophobic drugs. However, it was found that the modified starch was difficult to keep in solution due to the high molecular weight and hyper branched structure of the starch: it reprecipitated without stirring. Furthermore dissolving the polymer into organic solvent was found to take a long time[4]. In another study, T. Stauner also introduced hydrophobically-modified starch derivatives such as allyl- or phenyl-modified starch to make NPs. The modification requires NaH to be used with DMSO. Industrial translation of this process is not advisable as NaH contact with water is explosive [5]

In order to improve on the above mentioned problems with starch polymers, we focused on β-Cyclodextrin (β-CD) as an alternative starch which is a native material. β-Cyclodextrin (β-CD) is a cyclic oligosaccharide, consisting of seven glucose units linked by α-1,4-glucosidic bonds[6]. Because of the low toxicity of this natural sugar derivative, β-CD has shown promise in gene therapy and other biomedical applications[7-9]. Moreover it is easier to chemically modify than starch. Especially the 21 substitutable hydroxyl groups on the peripheral surface of β-CD provide the possibility to produce a functionalized β-CD: for example, star shaped polymers[10, 11], rotaxanes[12, 13] and amphiphilic materials[14].

The β-CD based star polymer, which is produced by controlled polymerization (CRP) process, has an advantage that the component of side chain is also controllable. For example, both hydrophobic side chain and amphiphilic side chain star polymers can be produced by CRP. By controlling side chain length and molecular weight distributions, star polymers showed good solubility in organic solvent compared to starch polymers. In addition, conventional radical polymerization or coupling

reaction with β-CD-core and side chain (graft on to process), CD star polymer produced by CRP has two more advantages. First, one can easily tune the hydrophobicity of the side chain by changing the component of monomer. Second, one can easily adjust the length of the side chain by changing the ratio of monomer and initiating groups[15-22].

In this study, we produced star polymers from the β-CD core with different kinds of side chains by the CRP method. From this material, NPs with hydrophobic actives were formulated to for drug delivery system applications (Scheme1).



Scheme 1. Producing star polymer with block polymer arm

2. Experimental

2-1. Materials

β-Cyclodextrin (β-CD) (CAVAMAX®W7) was obtained from Wacker Chemie AG(Stuttgart, Germany) and dried in vacuum at 80°C overnight just before use. Methyl methacrylate (MMA) (Aldrich M55909: 99%) and tert-butyl acrylate (t-BA) (Aldrich 327182; 98%) were obtained from Sigma-Aldrich (Munich, Germany) or BASF (Ludwigshafen, Germany) and purified by passage through a column of activated basic alumina to remove inhibitor[23]**.** All other reagents were obtained from Sigma-Aldrich (Munich, Germany) and used without further purification. Polyvinyl alcohol (PVA) Mowiol 4-88 from Kuraray Specialities Europe GmbH (Frankfurt,

Germany) and ethyl acetate from Fluka Chemie GmbH (Buchs, Switzerland) were used as obtained. Double distillated water was used to prepare nano particles.

2-2. Methods

Gel permeation chromatography (GPC) was performed as follows: Samples of the polymers were dissolved in the eluent (3 mg/mL), shaken for 16 h and injected by a 20 μL sample loop into a GPC system equipped with a refractive index detector Shodex RI-101 (Refractive Index Detector) with a flow rate of 1.0 mL /min. Data evaluation was performed by win GPC Unity Version 7.2 software from PSS (Polymer Standard Service.GmbH) Mainz, Germany. For the star polymers THF was used as the eluent with a Shodex PSS SDV 5 μm, 8.0*300 mm column (calibrated with narrow molecular weight poly styrene standards from PSS, Mainz, Germany)

UV spectra were taken with a UV/VIS spectrometer Lambda 2 (Perkin Elmer) in 0.1 or 0.5 weight % solution in THF or Toluene. IR spectra were taken by a tensor 27 FTIR spectrometer (Brucker, Germany) from powdered samples with a golden gate diamond ATR unit.

Size and zeta-potential of the nanoparticles were analyzed by dynamic light scattering (DLS) and electrophoretic mobility, respectively, using a Nano-ZS (Malvern Instruments, Malvern, UK).

The morphology of particles was examined by Atomic Force Microscopy using(AFM) a Nanoscope IV Bio-scopeTM (Veeco Instruments, Santa Barbara, CA, USA). Imaging was done using taping mode and a silicon cantilever with a spring constant of approximately 40 N/m and a resonance frequency of about 170 kHz. The scan speed applied was 0.2 Hz.

2-3. Cyclodextrin based Br-Macroinitiator. Same with Chapter II

2-4. General polymerization procedure[10, 11]

In general ATRP was preceded was same with chapter II

2-5. General procedure to prepare the NPs was same with chapter II

2-6. Cell culture is same with chapter II

2-7. MTT assay is same with chapter II

3. Results and Discussion

3-1. Producing the star polymer family by ATRP

Table 1. Producing the star polymer with block polymer arm chain.

Run [1)]	1st Monomer/ 2nd monomer [3)]		M_w	M_w/M_n
1[2)]	MMA / tBA =10/10	Random	3.0 x 10^4	1.54
2[2)]	MMA / tBA =10/10	Block	2.2 x 10^4	1.76
3	MMA / tBA =10/10	Block	2.0 x 10^4	1.32
4	MMA / tBA =10/10	Block	2.3 x 10^4	1.21
5	MMA / tBA =20/5	Block	2.6 x 10^4	1.25

1) MMA and tBA(1/1 or 4/1) / Macroinitiator / Catalyst / Tris-4(-Methoxyphenyl)-Phosphine = 400 or 500/ 20 / 20 / 40 mM in solvent at 80 °C
2) Polymerization in DMSO
3) Monomere conversion is between 65 and 85 %

Star shaped polymer with CD core and tBA-MMA random or block copolymer arm was produced by ATRP (Table1). The polymerization conditions were optimized by the authors previous studies and their producing processes were similar to mainly homo polymerization of MMA from CD, which were introduced by the authors in Chapter II of this thesis in detail.

By using the ATRP, not only the length of the arm chain but also the rate of MMA and tBA in the arm can be controlled. Run 1 and Runs 2 show the result of random- and block- copolymerization of MMA and tBA in DMSO. In case of the block copolymer, polymerization of 1st and 2nd monomers run smoothly and relative controlled molecular weight polymer was obtained in 24 hours. Solvent color was changed during the reaction from green to light white and molecular weight distribution become relative broad (M_w/M_n=1.6). In the UV spectum no peak was observed between 500 and 600 nm, which is the characteristic peak for Ni catalyst. It indicated that DMSO influenced the structure of the catalyst during the polymerization. This might be one reason that molecular weight distribution was getting broader.

Hence polymerization of MMA and tBA was examined in non polar solvent like toluene. Run 3 and Run 4 are the results of star polymers with block copolymer of MMA and tBA arm chain in toluene. In this reaction solvent kept the color during the reaction and well controlled polymer was obtained even after reaction. UV spectrum was observed between 500 and 600nm. The results of UV spectrum also supported

our hypothesis that Ni catalyst did not loss its activity therefore well controlled polymer was obtained.

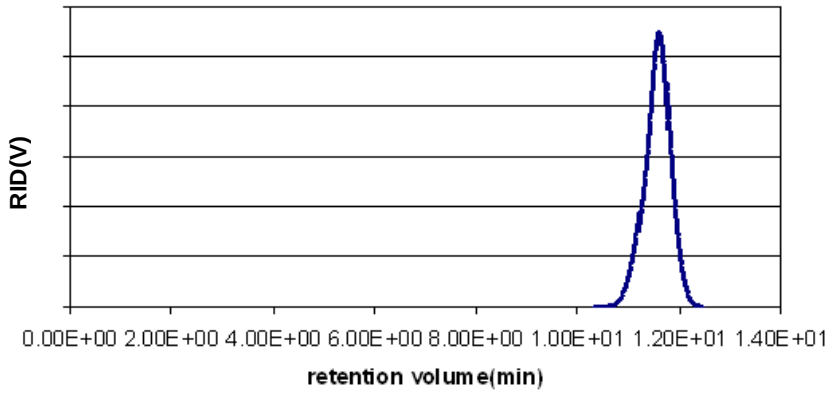


Figure 1. GPC curve of star polymer with block polymer arm (Run 4 in table 1)

Figure 1 shows the GPC-results of Run 4 trial. No side peak was observed in higher area. It indicates that Star-Star coupling did not occur during the polymerization. In addition any tailing peaks in lower area of GPC were observed in star polymers. It also suggested that block copolymerization was occurred smoothly without any side reactions. Reproducibility of same structure polymer is important especially for pharma applications to keep the quality of the drug formulations. But all natural polymers and polymers, which were produced by free radical polymerization, have the disadvantage of low reproducability. In contrast in case of the ATRP, it is easy to reproduce the same molecular weight and / or structure polymer by controlling the rate of initiator and monomer. Run 3 was produced 2 times to observe the reproductivity of ATRP (Run 3 and Run 4). In both trials, well controlled polymers were obtained. (M_w/M_n <1.3).

3-2. Amphiphilic star polymer

Amphiphilic star polymer was produced by hydrolyzing reaction of tBA to acrylic acid (AA). A new peak was observed around 3500cm^{-1} by IR measurement after the hydrolyzing reaction. It means that tBA group were sure to change to the AA group smoothly. Furthermore dramatically molecular weight decrease was not observed before and after the hydrolysis reaction. It also indicated that hydrolyzing reaction influenced only tBA parts of the polymer. Star polymer with amphiphilic block arm chain families are introduced in table 2. Run 6 were produced from polymer rune 3 in

table 1(M_w/M_n=1.27) and run 7 was produced from polymer run 5 in table 1(M_w/M_n=1.22).

3-3. Optimization of nanoparticle preparation

Nanoparticles with CD star polymers were produced by emulsion technique[4, 5]. If NPs are bigger than 250 nm it is difficult to reach cell uptake and if they are smaller than 50 nm the loading capacity gets very low. Therefore the particle size should be between 100 nm and 200 nm to apply for nano medicine applications in the future[24]. In order to use CD star polymers as carrier material for slow release systems for hydrophobic drugs in the future, nanoparticles with spherical shape without core-shell structure are the target of this study.

The colloidal properties of these particles were maintained by using PVA, which sterically stabilizes the suspension despite the very weak negative surface charge (zeta potential ~ -3 mV). Without PVA, the polymer alone did not possess enough amphiphilicity to maintain a stable nanoparticle suspension in water**.** We used PVA, which is already used in medical application, as an emulsifier to produce nanoparticles. The impact of the PVA concentration in the water phase was also examined. NP with star polymer (run 1 in table 1) was formulated with 0.1%, 0.5 % and 1% (wt/vol) PVA solution in water. Well size controlled NP were produced in all cases (table 2).

Table 2. Effect of concentrate of PVA to NP.(Mean ± SD, n=3)

	Particle size(nm)	PDI
1.0 % PVA	190.1±0.75	0.077 ± 0.022
0.5 % PVA	156.7±2.0	0.038 ± 0.012
0.1 % PVA	179.5±1.17	0.03 ± 0.023
0 % PVA	No NP	No NP

Even with a reduced amount of PVA, star polymer was able to produce well controlled NP dispersions. The possibility of PVA reduction may be a tool to modify the time needed for biodegradation of the NPs.

To evaluate the impact of the homogenization step, we prepared plain NPs using different stirring speeds (Figure 2, star polymer 1 in table 1 was used).

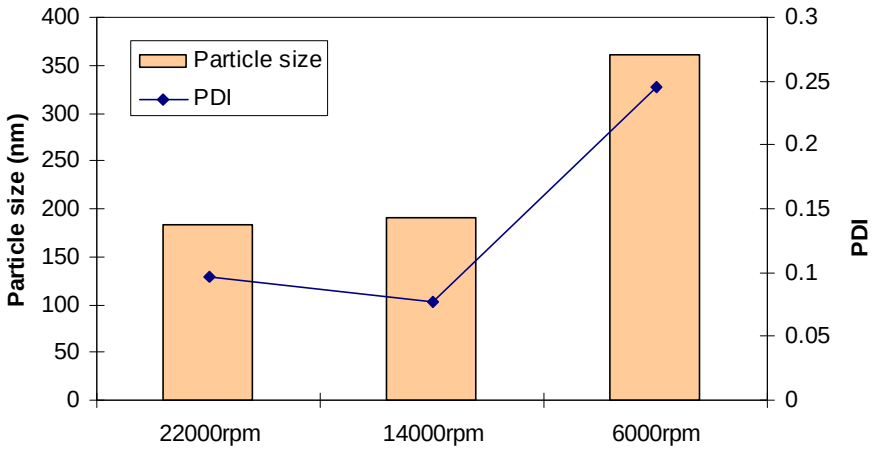


Figure 2. Effect of stirring speed to the size of NPs (Star polymer 1 in table 1) .(n=1).

Resulting particle size and PDI are shown in Figure 2. There seems to be a lower limit for optimum agitation at about 14000 rpm. Below this speed the particle size increase and the particle size distribution gets broader. However, increasing the homogenization speed further did not result in a further improvement neither of particle size nor of polydispersity.

Thereafter we analyzed the influence of polymer concentration in the organic phase in the range of 4 to 0.5 mg polymer/mL. To eliminate possible errors caused by high particle concentrations on the measured mean hydrodynamic diameter of particles we diluted the particle suspension to the final concentration of 0.1mg NP/mL with purified water before light scattering measurement. There was no significant effect of the polymer concentration on particle size and poly dispersity of the particle suspension (table 3, star polymer 1 in table 1 was used).

Table 3. Impact of polymer concentration on NP size(Mean ± SD, n=3).

4 mg polymer/ml					
Undiluted		*1+1 dilution*		*1+3 dilution*	
Particle Size(nm)	*PDI*	*Particle Size(nm)*	*PDI*	*Particle Size(nm)*	*PDI*
183.8±1.12	0.017±0.006	170.5±0.68	0.053±0.004	165.2±0.643	0.056±0.013

2 mg polymer/ml			
Undiluted		*1+1 dilution*	
Particle Size(nm)	*PDI*	*Particle Size(nm)*	*PDI*
182.9±2.52	0.065±0.02	171.2±1.67	0.072±0.019

0.5 mg polymer/ml	
Undiluted	
Particle Size(nm)	*PDI*
162.1±0.53	0.0783±0.031

However looking for a system to encapsulate idarubicin we chose a polymer concentration of 1mg polymer/mL as standard condition for the further experiments. The low solubility of idarubicin base in ethylacetate limits the drug loading. Using higher polymer concentrations would further decrease the loading defined as percent of weight drug to weight NP.

3-4. Nanoparticle study; effect of hydrophobicity to NP

Table 4. Effect of polymer side chain with various hydrophobicity on NP size(Mean ± SD, n=6).

Run	1st Monomer/ 2nd monomer		Particle Size(nm)	PDI
CD-1	MMA / tBA =10/10	Block	167.7±3.18	0.109±0.02
CD-2	MMA / AA = 10/10	Block	158.7±20.8	0.304±0.05
CD-3	MMA / tBA = 20/5	Block	160.6±1.97	0.165±0.03
CD-4	tBA / MMA = 20/5	Block	138.8±4.04	0.11±0.014

Hydrophilic modified star shaped polymer was produced by ATRP of tBA and hydrolysed by tri-fluoroacetic acid mildly as a reference (CD-1 and CD-2). NPs with CD-2 was not enough hydrophobic to produce well controlled NPs (Particle size is

158.7 nm, PDI = 0.3) because it is to hydrophilic to stabilize the core of nano particles. Thereafter MMA-tBA arm chain hydrophobic modified cyclodextrin star polymers were produced, because it played an important role to stabilize the core of nanoparticles as well as to increase the hydrophobicity of the core (CD-1). Nanoparticles with star polymer, which had the same length and the opposite order of arm chain, were produced (CD-3 and CD-4). The results indicated that the order of PMMA and tBA does not influence the particle size and PDI. But the hydrophobicity of polymer influenced dramatically to the size and PDI of NP. Thereafter we concluded that hydrophobic modified cyclodextrin is suitable to produce NP.

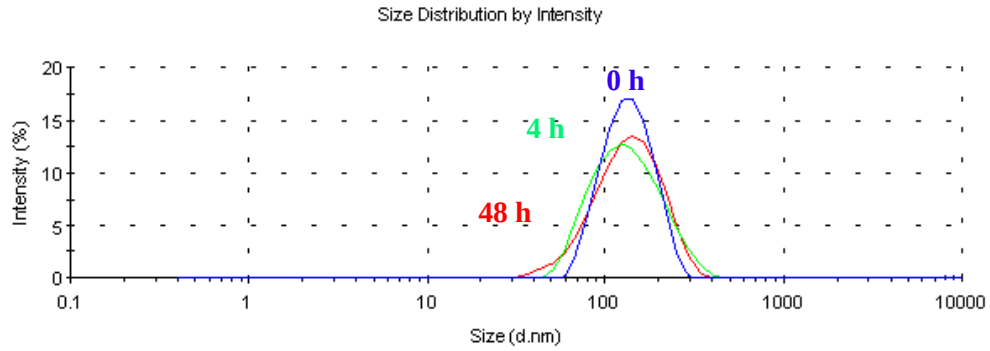
3-5. Nanoparticle study; effect of pH to NP stability

Table 5. Storage stability of NP at 4°C.(Mean ± SD, n=3)

	Nanoparticle		after 3 weeks		after 6 weeks	
Star polymer	Size(nm)	PDI	Size(nm)	PDI	Size(nm)	PDI
Run 1. in Table1	190.1± 0.75	0.077 ± 0.022	191.5± 2.16	0.084± 0.032	188.2± 0.21	0.078± 0.0072

Table 5 indicated that all polymers were enough hydrophobic to produce well controlled nano particles (PDI is smaller than 0.2) with mono modal size distribution. In order to use nanoparticles as carrier for drugs it is important to observe their stability in aqueous media and at various pH values. Therefore produced NPs were stored by 4°C for 6 weeks. Even after 6 weeks NP kept their initial size and PDI (PDI<0.2). NP were stable in neutral water conditions. As a drug carrier application, collapse of NP in different pH is also important factor, hence, stability of NP was observed in the model stomach condition (pH 2 at room temperature) additionally. Figure 3 shows the results of this stability test from two different NPs. NP was still stable after 48 hours in case of the NP with a short tBA arm star polymer (star polymer 6). But in case of the NP with a long arm tBA star polymer (star polymer 5), NP lose its stability in 48 hours and PDI was getting broader (figure 3). This result indicated that tBA was hydrolysed in 48 hours and NP lost its stability. In this study we learned that by changing the rate of tBA chain we are able to produce pH sensitive NP. The use of a pH-sensitive system may be of high interest for various medical applications.

a) NP with star polymer 5



b) NP with star polymer 6

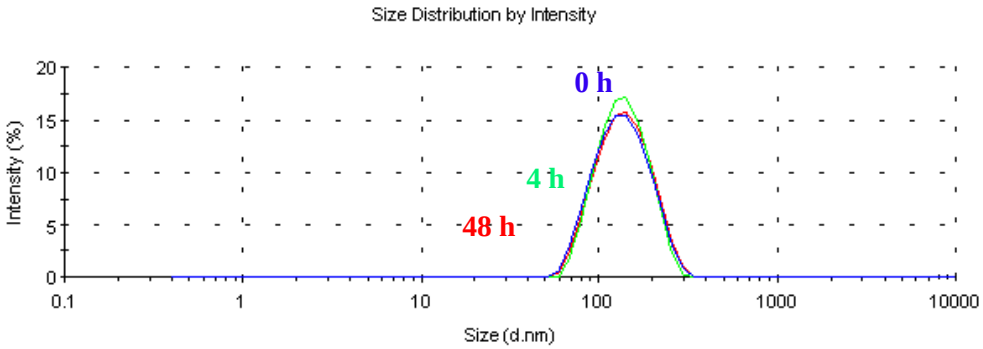


Figure 3. Particle size distribution of starpolymer 5 and 6 NP following incubation at pH2.

However in case of MMA and tBA random copolymer arm star polymer, it does not lose its stability in acid conditions. Then we concluded that by tuning the composition of the arm of star polymer, we found a possibility to control the stability of NP by this study.

Furthermore, strategies to produce NPs without the addition of PVA were also investigated by using the amphiphilic arm star polymer. These results also will be introduced by authors soon.

3-6. Nanoparticle study; toxicity

Cytotoxicity was analysed using MTT assay, which monitors the mitochondrial metabolism of cells as an indicator of their viability.

Figure 5 displays the MTT test results after a 3 hour incubation period with the star polymer nanoparticles. The tests demonstrated that concentrations up to 0.333 mg NP/ml are not cytotoxic in the Caco-2 test model. A deviation of some percent in viability is considered normal in cell culture. The standard deviation of the untreated

cell controls is indicated as dashed lines. The observed results for all tested concentrations are within the standard deviation (Figure 4).

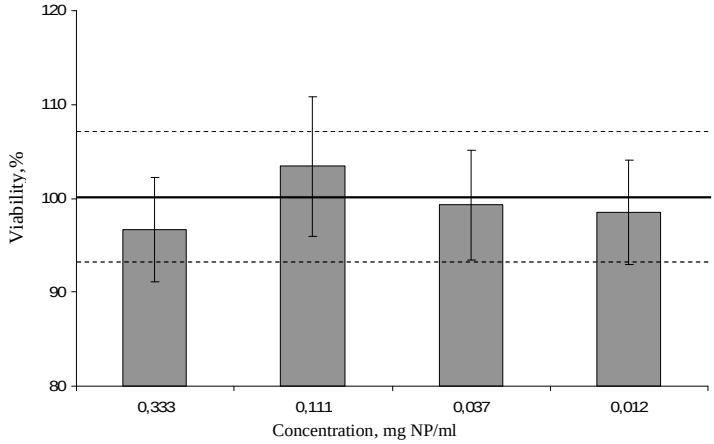


Figure 4. Cytotoxicity test of CD star polymer particles(run 1 in Table 1)in Caco-2 cell culture.(Mean ± SD, n>3).

4. Conclusion

MMA-t-BA block copolymer arm chain star polymer (hydrophobe-type)and MMA and AA copolymer arm chain (amphyphilic type) star polymers, whose composition and length of the arm chain were varied, were successfully produced by Nickel catalyzed ATRP. Monomer conversions were achieved over 50 % and star-star coupling were inhibited in this reaction. Therefore obtained polymers were homogenious in molecular weight as could be proofed by GPC analysis (M_w / M_n<1.3). Best particles were achieved with star polymer with MMA and tBA random copolymer. The optimized nanoparticle producing procedure was as following, 1mg polymer in 1mL ethyl acetate 1400 rpm stirring speed with 1% PVA as an emulsifier(size 190nm, PDI=0.77). Main influence factor for particle size distribution and stability against pH2 conditions (model stomach) was the composition of the MMA and tBA arm chain. By tuning the composition of the arm of star polymer, we demonstrated the possibility to control the stability of NPs to achieve a “tailor made system”. Release kinetics measurement of Idarubcine will be examined as a next step.

5. Acknowledgement

This research financially supported by BASF SE and BMBF (Bundesministerium für Bildung und Forschung, Project No. 13N9131). We would like to appreciate to T. Stauner, M. Keil, C. Thiele, J. AX, J. Ganz and B. Bossmann regarding for assistance.

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IV. Grafting PMMA from Starch by ATRP and Formation of Nanoparticles for Advanced Drug Delivery

Abstract

The aim of this study was to synthesize hydrophobic starch based graft polymers by atom transfer radical polymerization (ATRP) to provide new biocompatible nano-carriers for the delivery of hydrophobic drugs. Waxy corn starch, partially degraded by α-amylase, of a typical molecular weight of 20,000 g mol^{-1} was partially (DS 0.34) esterified with α-bromoisobutyryl bromid. The resulting starch α-bromoisobutyrate was used as a macroinitiator for the ATRP of methyl methacrylate (MMA) at 80°C in DMSO. Ni(II) triphenylphosphine complexes were found to be catalysts advantageous to the well known Cu(I) complexes, because they are much better to remove from the product after polymerization. Graft polymers of a typical molecular weight of 5.75 10^5 g mol^{-1}, equivalent to an average graft DP 70, were obtained after a monomer conversion of 70%. Since these graft polymers are soluble in organic solvents like THF and ethyl acetate, nanoparticles can be formed in water / ethyl acetate using the emulsion-diffusion technique with polyvinyl alcohol as the emulsifier. The resulting particles showed spherical shapes, sizes below 200 nm and narrow size distributions (PDI<0.18) by dynamic light scattering and atomic force microscopy. Since cell viabilities remain unaffected by these nanoparticles, they are good candidates for the delivery of drugs.

1. Introduction

Polymer nanoparticles have attracted increasing attention over the past years for the delivery of active substances. Polymer nanoparticles are applicable for advanced formulations of drugs, cosmetics, sun protection, health food, veterinary medicine or plant protection products. The development of nanoparticles for the controlled release of drugs, has improved the therapeutic methods in the recent years. Nanoparticles can improve the uptake and the plasma level of sparingly soluble pharmaceutical agents. Drug delivery systems for cancer therapeutics using polymer nanoparticles have revolutionized medicine. Delivery systems have improved the efficacy and reduced the toxicity of current therapies and resulted in the development of new ones. Targeted delivery systems of chemotherapeutics to the tumor compartment can be achieved systemically, either passively or actively. Nano-incapsulation radically changes the pharmacokinetics of the included drug, and nanoparticles target tumors passively via the enhanced permeability and retention (EPR) effect.[1-4] Several synthetic polymers such as polyacrylates,[5] PEG- blockcopolymers,[6-9] polylactide-glycolide copolymers,[10-12] polycyanacrylates[13], and polycaprolactone[14] were already used as matrices for nanoparticle formation. Main drawbacks of using these synthetic polymers are poor biodegradability and too high crystallinity leading to a hampered release of the drug. As a consequence, biopolymers, such as proteines,[15] chitosan,[16, 17] or modified biopolymers, like crosslinked saccharides,[18] cyclodextrin derivatives,[19] polycaprolacton-dextran,[14, 20] polyglycolide-lactide-dextran[21], cholesterol-pullulan[22] and starch derivatives[23-27] were used as well for the formation of nanoparticles. Recently, we demonstrated that nanoparticles produced from propylated starch are well suited for controlled release formulations of active substances like caffeine.[28] On one hand, nanoparticles from biopolymer derivatives are easier to obtain and are more stable than the ones from native biopolymers, since hydrophobicity can be increased by derivatization. On the other hand, extensive derivatization leads to reduced biocombatibility and biodegradability which might cause problems in the later administration of them. In the following we describe how to avoid these problems by the synthesis of hydrophobic starch derivatives with low degree of substitution by atom transfer radical polymerization (ATRP) and by formation of biocompatible nanoparticles with the well-established emulsion-diffusion technique.

In the past, starch was already grafted with alkyl acrylates with conventional free radical polymerization using ceric ammonium nitrate as initiator.[29] The main

drawbacks of this method is poor structural control and the toxicity of cer, which might remain in the product. Grafting of polymer chains from polymeric initiators by ATRP offers the advantage of yielding branched polymers with well-defined structures and low polydispersities.[30, 31] ATRP with copper catalysts was already used for grafting cellulose,[32,33] cyclodextrins,[34,35] maltoheptaose,[36] as well as starch.[37, 38] Due to the inherent poor solubility of starch, ATRP grafting was performed either under heterogenous conditions,[37] or in solution with a soluble starch acetate.[38] The use of copper catalysts is problematic, because copper ions tend to stick in the polysaccharide backbone due to complex formation. Therefore they are difficult to remove from the product after polymerization. Due to their higher stability and lipophilicity Ni^{2+} triphenylphosphine complexes should be advantageous to copper catalysts in this respect. From the work of Sawamoto et al. these Ni^{2+} triphenylphosphine complexes are indeed already known for ATRP of methacrylates, but were never used for grafting starch.[39-43] In the following we describe ATRP from α-bromoisobutyrates of native starch with Ni catalysts under homogenous conditions, according to Figure 1, leading to well-defined starch PMMA comb polymers and the formation of nanoparticles in water suited for drug delivery.

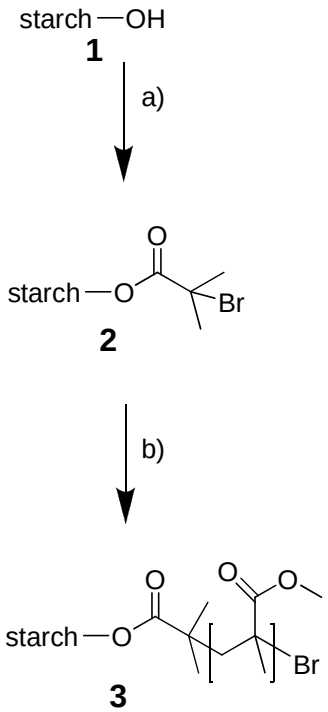

Figure 1. Synthesis of starch PMMA graft polymers by ATRP, a) α-bromoisobutyryl bromide, pyridine, DMF, b) MMA, $NiBr_2L_2$ (with L tris(4-methoxyphenyl)phosphine), DMSO.

2. Experimental

2-1. Materials

Waxy corn starch (100% amylose content) was received from Roquette (Germany) it was dried at 60 °C overnight before use. α-Amylase from bacillus licheniformis (EC 3.2.1.1 activity 120 KNU/g), and isoamylase from pseudomonas (E.C. 3.2.1.68 activity 3,000,000 un/mg) were purchased from Aldrich and stored at 4 °C.

Methyl methacrylate (MMA) was purchased from Aldrich and purified by filtration through an alumina column to remove the inhibitor and stored at 4 °C. Polyvinyl alcohol (PVA) Mowiol 4-88 from Kuraray Specialities Europe GmbH (Frankfurt, Germany) and ethyl acetate from Fluka Chemie GmbH (Buchs, Switzerland) were used as obtained. Double distillated water was used to produce NP. Solvents *N*,*N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO) and tetrahydrofurane (THF) were obtained from BASF and stored over sieves (0.3 nm, Carl Roth, Germany) and under N_2. Calcium acetate, α-bromoisobutyryl bromide, $NiBr_2(PPh_3)_2$ and tris(4-methoxyphenyl)phosphine (Aldrich) were used without further purification.

2-2. Methods

Gel permeation chromatography (GPC) was performed as follows. Samples of the polymers were dissolved in the eluent (3 mg/mL), shaken for 16 h and injected by a 20 μL sample loop into a GPC system equipped with a refractive index detector Shodex RI-101 (Refractive Index Detector) with a flow rate of 1.0 mL /min. Data evaluation was performed by win GPC Unity Version 7.2 software from PSS (Polymer Standard Service.GmbH) Mainz, Germany. For the star polymers THF was used as the eluent with a Shodex PSS SDV 5 μm, 8.0*300 mm column (calibrated with narrow molecular weight poly styrene standards from PSS, Mainz, Germany)

UV spectra were taken with a UV/VIS spectrometer Lambda 2 (Perkin Elmer) in 0.1 or 0.5 weight % solution in THF. IR spectra were taken by a tensor 27 FTIR spectrometer (Brucker, Germany) from powdered samples with a golden gate diamond ATR unit. NMR spectra were recorded by a Bruker Avance 500 spectrometer (1H: 500.00 MHz, 13C: 125.71 MHz) using tetramethyl silane as the internal standard 0.00 ppm.

Size and zeta-potential of the nanoparticles were analyzed by dynamic light scattering (DLS) and electrophoretic mobility, respectively, using a Nano-ZS (Malvern Instruments, Malvern, UK). The morphology of particles was examined by Atomic Force Microscopy using a Nanoscope IV Bio-scopeTM (Veeco Instruments, Santa Barbara, CA, USA). Imaging was done using taping mode and a silicon cantilever with a spring constant of approximately 40 N/m and a resonance frequency of about 170 kHz. The scan speed was 0.2 Hz.

2-3. Synthetic procedures

Partially degraded starch 1. α-Amylase (53 mg) was dissolved in 1.2 L aqueous 70 mM calcium acetate solution, and waxy corn starch (300 g) was added slowly under the stirring at room temperature. After the pH was adjusted to 6.5 by addition of 5M NaOH the temperature was raised to 90 °C. After 4 h the pH was set to 3.5 by addition of 10% HCl at 90 °C in order to deactivate the enzyme. Afterwards, pH was adjusted to 7.0 by addition of 5M NaOH. The resulting solution was filtered through the porous glass filter funnel loaded with one layer (height 0.5 cm) of silica gel Si-60 (Aldrich). The filtrate was concentrated in vacuum at 60 °C. The solid white residue was dried in a vacuum oven at 60 °C for 24 h (267 g, 89 %). The aqueous solubility of the degraded starch was 30 wt. % at 25°C. Peak molecular weight by GPC: 20,000 g/mol.

α-Bromoisobutyryl starch 2. A mixture of degraded starch 1 (4.05 g , 0.025 mol per glucose unit), pyridine (6.33 g (0.08 mol) in 100 mL DMF was stirred for 1 h at 25 °C. Then a solution of α-bromoisobutyryl bromide (18.32 g, 0.08 mol) in 100 mL DMF was added. The reaction mixture was stirred for 6 h at 80°C under N_2. After cooling to r.t. the product was precipitated in 1 L water. The yellow solid product was isolated by filtration and washing with 1 L water. The crude product was dissolved in ethyl acetate (concentration 5 wt. %) ultrafiltated over a Selro® MPF-36 membrane (Koch Membranes, Aachen, Germany). Yield 1.37g (30%). Elemental Analysis, found C 39.5%, H 6.6%, Br 12.6%, calculated for DS 0.33 C 41.5%, H 5.7 %, Br 12.5%. ^{1}H NMR (400 MHz, d^6-DMSO) δ 1.90 (s, CH_3) , 3.5-5.5 ppm (m, 70H, starch).

Starch-MMA graft polymers 3. A solution of $NiBr_2(PPh_3)_2$ (149 mg 0.2 mmol), tris(4-methoxyphenyl)phosphine (140 mg 0.2 mmol) in 10 mL DMSO was stirred over night in a baked 3 neck flask under N_2. Afterwards methyl methacrylate (2 g, 20 mmol) and 2-bromosisobutyryl starch **2** (16.2 mg, 0.1mmol per glucose unit and 0.034 mmol Br initiating group) was added and stirred for 24 h at 80°C under N_2. Conversion of samples was determined from the concentration of residual monomer relative to 1,2-dichlorobenzene (internal standard) by ^{1}H NMR. After termination by cooling with an ice bath and exposure to air, the resulting mixture was evaporated to remove solvents and diluted with dichloromethane. It was extracted 3 times with acidic water and furthermore precipitated in water. Polymers were dried over night in under vacuum at 65°C and finally obtained as white powders.

2-4. Preparation of nanoparticles

A solution (1 mL) of the starch PMMA graft polymer **3** in ethyl acetate (1 gL^{-1}), 1 mL was dropped into 4 mL of an aqueous polyvinyl alcohol solution (1% w/v). This biphasic system was agitated with a high speed homogenizer (Ultra Turrax® Ika®, Brasil Ltda, Taquara, Brasil) at 14000 rpm for 15 min. Then, 5 mL water (MilliQ) was added and stirred for further 16 h in an open fume hood at r.t. to allow evaporation of ethyl acetate.

2-5. Cell viability Assay (MTT)

Caco-2 cell line clone C2BBe1 (ATCC No CRL-2102) passage no 70 was used as test system. DMEM No E-15-810 (PAA, Pasching, Austria) supplemented with 1% non-essential amino acids (Gibco, Karlsruhe, Germany) and 10 % FBS (No P30-3300, PAN Biotech, Aidenbach, Germany) was used as growth medium. Subculture was done once a week at a subcultering ratio of 1:10 using trypsin-EDTA (No 25300, Gibco; Karlsruhe, Germany). Thiazolyl Blue Tetrazolium Bromide (M5655, Sigma Aldrich) was dissolved in PBS pH 7.4 to yield a final concentration of 5 mg/ml for the stock solution. Cells were seeded at a density of 10,000 cells per well in a 96-well cell culture plate. After five days propagation confluence was reached and MTT assay performed. Before exposure to particle suspension cells were washed twice with Krebs-Ringer buffer pH 7.4 (KRB). Particle suspension was separated by centrifugation of the primary particle suspension to remove the water and re-suspension in KRB supplemented with 10% FBS. Samples for the concentrations of

0.333 to 0.012 mg NP/ml were exposed to the cells. After the incubation period of 3 h the particle suspension was removed. Cells were washed once with KRB before fresh medium with MTT reagent (0.5 mg/ml) was added. After further 4 h incubation 100 µl lysis buffers (10% SDS in 0.01mM HCl) were added to lyse the cells and solubilize the tetrazolium crystals. The absorbance at 550 nm was analysed in a plate reader. Viability was calculated in comparison to the negative control, untreated cells as 100% value, and positive control 1%-trition solution as zero% value. In parallel a cell-free assay with the same particle concentrations was performed to ensure that the colorimetric assay was not affected by the interactions with the nanoparticles.

3. Result and Discussion

3.1 Synthesis of the starch-PMMA graft polymer

Because of its high molecular weight and its highly branched molecular structure, derivatization of native starch was difficult because of high viscosities. Also the esterification was poorly reproducible and products were too heterogenous. In order to overcome these difficulties, various starches were degraded by α-amylase or a mixture of α-amylase and *iso*-amylase. α-Amylase is known to cut the glycosidic 1→4 bonds of starch, while *iso*-amylase cleaves the 1→6 bonds.[43] The obtained starches S2 to S6 in table 1 are highly water-soluble (30% in distilled water by room temperature), their molecular weights are summarized in Table 1. Degraded starch obtained for reaction conditions S4 was found most appropriate for subsequent derivatization after removal of maltooligosaccharides by ultrafiltration.

Table 1. Enzymatic degradation of native starches

Entry	**Type of Starch**	**Amount of amylase**
Natural Starch	waxy corn	-
S1	waxy corn	α-amylase 0.0025% iso-amylase 0.01%
S2	waxy corn	α-amylase 0.01%[1)]
S3	waxy corn	α-amylase 0.02%[2)]
S4	waxy corn	α-amylase 0.02%[1)]
S5	Corn	α-amylase 0.02%[1)]
S6	Potato	α-amylase 0.02%[1)]

1) Reaction time 4 h, 2) Reaction time 2 h

The starch based ATRP macroinitiator **2** was synthesized by esterification of the partially degraded starch **1** with α-bromoisobutyryl bromide under completely homogenous conditions in pyridine / DMF. Starch α-bromoisobutyrate **2** (DS 0.34) was isolated by ultrafiltration as a white powder soluble in organic solvents like

DMSO, Acetone, DMF and NMP. ATRP of MMA from macroinitiator **2** had to be performed in DMSO solution since starch derivative **2** was not soluble in less polar solvents such as toluene or cresol often used for ATRP. In addition DMSO is a favorite solvent for biomedical applications, since it is regarded as nearly non toxic. Nickel catalysts were chosen instead of the common copper catalysts because they are easier to remove from the reaction product than copper catalysts. Tris(4-methoxyphenyl)phospine was taken as the ligand. In comparison to other aryl phosphines it leads to the highest catalytic activity. The Ni complex was prepared by ligand exchange of $NiBr_2(PPh_3)_2$ with tris(4-methoxyphenyl)phospine.

Polymerizations were performed with a molar concentrations of MMA, **2**, $NiBr_2(PPh_3)_2$, tris(4-methoxyphenyl)phospine of 4000 : 20 : 20 : 40 mM at 80°C. Concentrations have to be kept low to avoid cross-linking. Kinetics of the polymerization, shown in Figure 2, was detected by ^{1}H NMR. High conversions of 70% could be reached after 1 d without cross-linking. At 40°C the final conversion was much lower (11%). The kinetics was 1st order respective to MMA concentration indicative for a living mechanism. The molecular weight M_w of the resulting starch-PMMA **3** was 5.75 x 10^5 g mol^{-1}. The polydispersity M_w/M_n = 1.77 was higher than for other ATRP polymers which was attributed to the high polydispersity of the starch backbone.

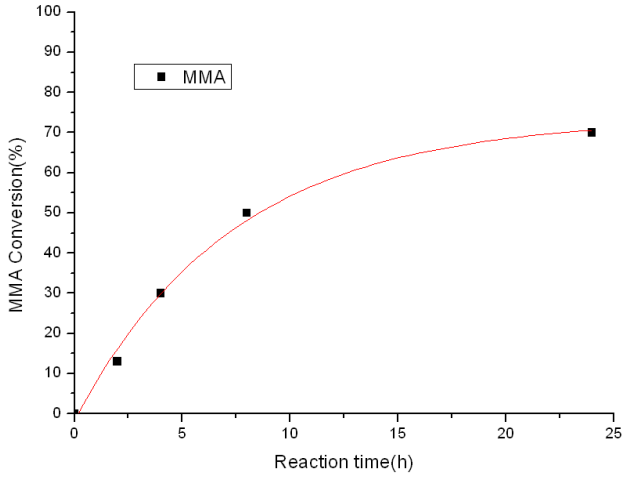

Figure 2. Polymerization of MMA from macroinitiator S 4 at 80°C in DMSO.

Polymer **3** was soluble in many organic solvents, like ethyl acetate, THF, Acetone, CH_2Cl_2 and DMF. Since the toxic phosphines and the Ni catalyst could be removed

completely by liquid/liquid extraction, polymer **3** could be useful for drug delivery application.

3.2 Formation of nanoparticles

The addition of MMA arms to starch resulted in the formation of graft polymers that are hydrophobic enough to form nanoparticles The obtained particles were in a size range below 200 nm (180.6 nm mean hydrodynamic diameter) with a uniform monomodal size distribution indicated by a PDI of 0.154. The colloidal properties of the particles were maintained by using PVA, which sterically stabilizes the suspension despite the very weak negative surface charge (zeta potential ~ -3 mV). AFM image and result of light scattering analysis from starch-MMA graft polymer was observed, in order to use this NP as a drug carrier material in the future (Figure 3).

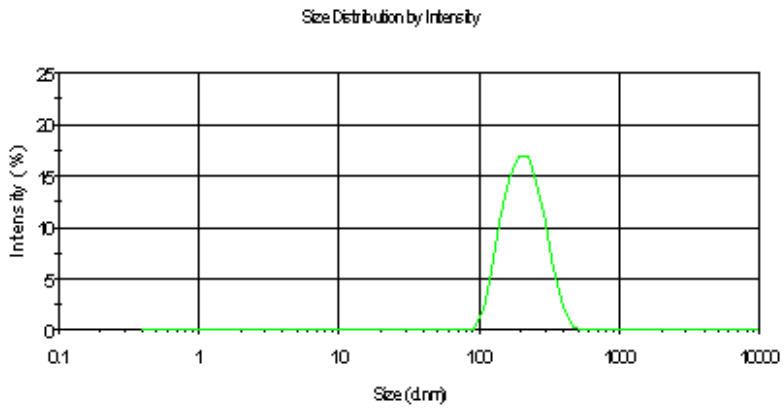

Figure 3. Particle size distribution of nanoparticles of starch-PMMA graft polymer **3** as determined by DLS.

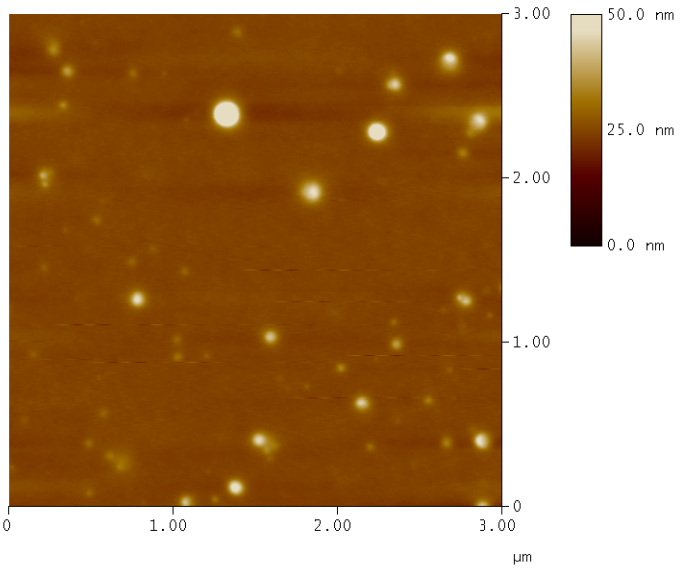

Figure 4. AFM pictures of nanoparticles of starch-PMMA graft polymer **3.**

Figure 4 illustrates the spherical shape of the NPs and their smooth surface. These modified starches represented a promising platform in the area of nanoparticle carriers. In order to apply these nanoparticles into a drug carrier system for hydrophobic drugs, flufenic acid (FFA) was used as a test active compound. Comparison of the particles with FFA and without FFA, reveals little differences in respect of both particle size and PDI. A narrow particle size distribution was maintained after incorporation of FFA (see Figure 5).

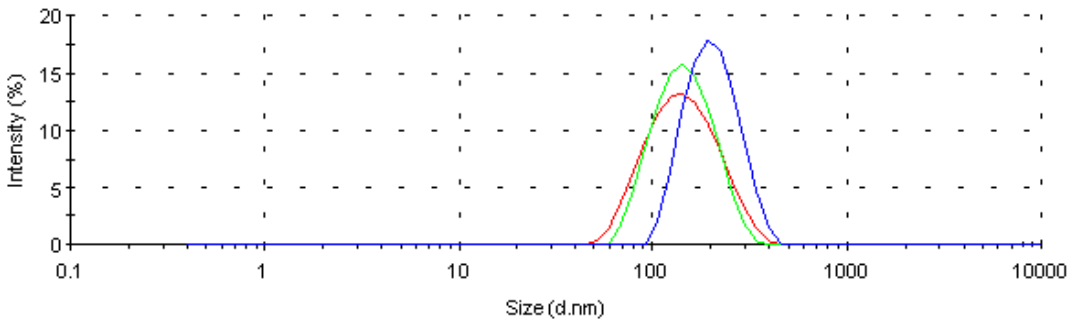

Figure 5. Nanoparticle size distribution. Blue line is a NP without FFA. Green line is a line with a FFA and Red line is a NP with FFA after 1 week.

3.3 Cell viability tests

Cytotoxicity was analysed using MTT assay, which monitors the mitochondrial metabolism of cells as an indicator of their viability. Figure 6 displays the MTT results after a 3 hour incubation period with the polymer **3** nanoparticles. The tests demonstrated that concentrations up to 0.333 mg $NPmL^{-1}$ are not cytotoxic for the Caco-2 test model. A deviation of some percent in viability is considered normal in cell culture. The standard deviation of the untreated cell controls is indicated as dashed lines. The observed results for all tested concentrations are within this standard deviation.

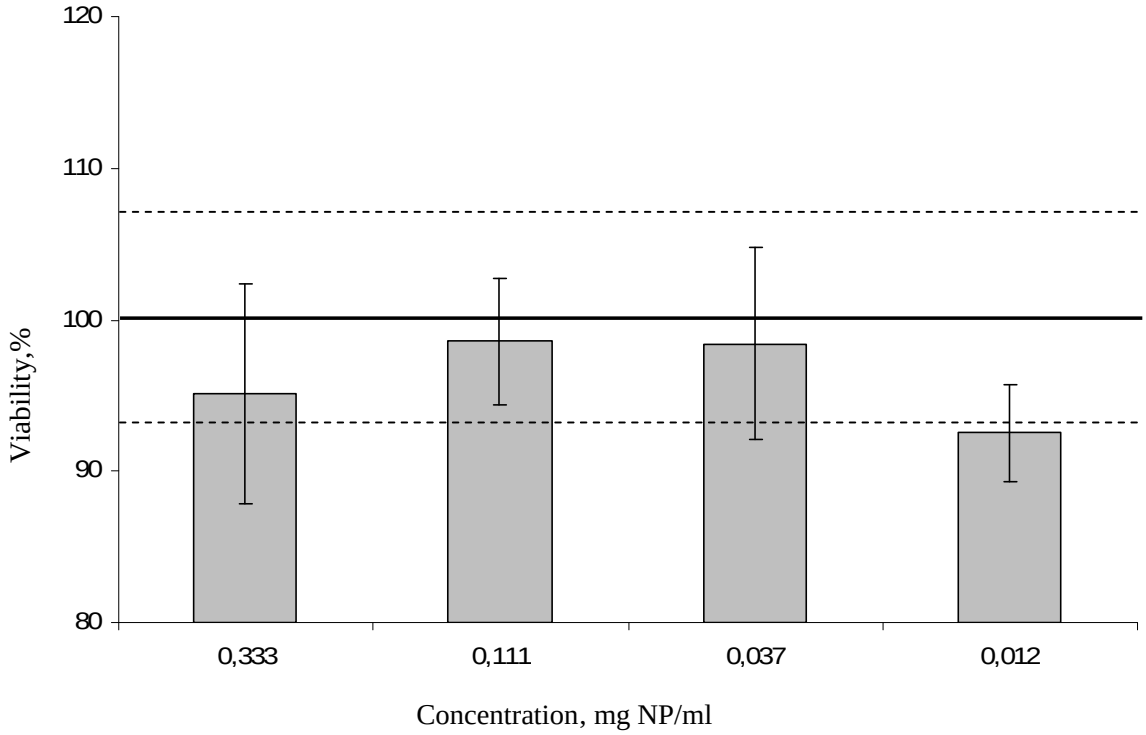

Figure 6. Cytotoxicity test of particles in Caco-2 cell culture. Toxicity was assessed after 3 h incubation time using MTT assay to monitor mitochondrial activity.(Mean ± SD, n=2).

4. Conclusion

Graft polymers with enzymatically decomposed starch as the main chain and PMMA side chains with controlled molecular weights were produced by ATRP. Conversions of up to 70% of the MMA could be reached without noticable crosslinking of the polymer.These graft polymers were enough hydrophobic to offer a promising platform for preparing nanoparticle drug delivery systems. The MMA-arms were expected to increase the encapsulation efficiency of actives within the nanoparticles

and allow controlled drug release. Nanoparticles prepared by the emulsion-diffusion technique exhibit small sizes (<200 nm), and uniform spherical shapes and narrow size distributions (PDI=0.12-0.18). Since cell viabilities remain unaffected by these nanoparticles, they are good candidates for the delivery of drugs.

5. Acknowledgement

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V. Synthesis and Characterization of modified Cyclodextrin derivatives for Nanoparticles

Abstract

Three different hydrophpbic modified cyclolodextrins were produced in conjunction with acid chloride in n-methyl pyrrolidone(NMP) at 40°C using cyclohexancarbonicacidchloride, phenylacetylchloride or 2-Bromoisobutyryl bromide as reagents. Obtained cyclodextrin macromonomers were used for nanoparticles (NP) formulations prepared by emulsion-diffusion method. Particle size was determined by dynamic light scattering (DLS) and particle morphology was evaluated using Atomic force microscopy (AFM).

Macromonomers were hydrophobic enough to produce monomodal distributed NP dispersions according to the poly dispersity index (PDI) of <0.08. Even after six weeks storage at 4°C the aqueous NP dispersion was stable with regard to particle size and size distribution. The AFM images showed round shaped particles with smooth surface but with a population of smaller particles which could not be seen in the dynamic light scattering.

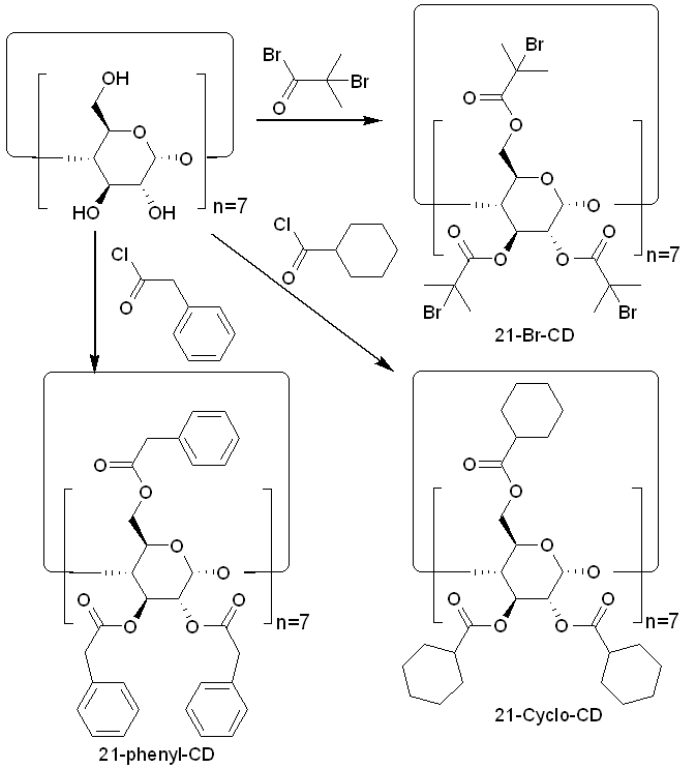
From this preliminary results the new hydrophobic modified CD seem to be promising candidates for encapsulation of active ingredients.

1. Introduction

Nanoparticles are solid, colloidal particles in a size range between 10 and 200 nm consisting of e.g. inorganic materials or macromolecular substances. In recent years nanoparticles have attracted increased attention in a variety of fields of application including catalysis, coatings, pharmaceutics, cosmetics, electronics and polymers[1-5]. The development of nanoparticles as drug delivery systems for controlled release of drugs has improved the therapeutic methods. Besides, incorporation of agents in micelles, liposomes, nano-capsules or nanoparticles[6]. These systems were used to improve the bioavailability in particular of sparingly soluble actives. Although many different polymers were used to produce nano-carriers there is still room for improvement in terms of biodegradability and well controlled polymerization product achievement.

β-Cyclodextrin (CD) is a cyclic oligosaccharide consisting of seven glucose units linked by α-1,4-glucosidic bonds[7]. Due to the low toxicity of this natural sugar derivative β-CD was used in gene therapy approaches and other biomedical applications, where it demonstrated promising results[8, 9]. In particular the 21 substitutable hydroxyl groups on the peripheral surface of β-CD provide the possibility to produce functionalized β-CDs such as star shaped polymers[10, 11], rotaxanes[12, 13] and amphiphilic materials[14]. In this work β-CD was used as core of star shaped macromolecules, whose –OH group was replaced to hydrophobic groups.

In our previous study we produced low toxicity nano particles with CD star polymer, which is produced by atom transfer radical polymerization (ATRP)[10, 11, 15]. However, due to the mass of necessary producing processes and high costs of ATRP catalyst, its industrialization was difficult. Therefore the development of an alternative simple process to produce hydrophobic polymers, which are acceptable for nanoparticle applications, is an ongoing challenge for industry. The aim of this study was to produce different types of hydrophobic modified CD like CD-star polymer in a simple process as is depicted in Scheme 1.and test them for nanoparticle preparation. The aim was to find a suitable polymer resulting in nanoparticle dispersions with a narrow size distribution and low toxicity.



Scheme 1. Reaction scheme of the modification of CD to produce various hydrophobic CD macro

2. Experimental

2-1. Materials

β-Cyclodextrin (β-CD) (CAVAMAX®W7) was obtained from Wacker Chemie AG (Stuttgart, Germany) and dried in vacuum at 80°C overnight just before use. N-methyl pyrrolidone (NMP) was obtained from BASF(Ludwigshafen, Germany). All other reagents were obtained from Sigma-Aldrich (Munich, Germany) and used without further purification. Polyvinyl alcohol (PVA) Mowiol 4-88 from Kuraray Specialities Europe GmbH (Frankfurt, Germany) and ethyl acetate from Fluka Chemie GmbH (Buchs, Switzerland) were used as obtained. Double distillated water was used to prepare nano particles.

2-2. Methods

IR spectra were taken by a tensor 27 FTIR spectrometer (Brucker, Germany) from powdered samples with a golden gate diamond ATR unit.

Size and zeta-potential of the nanoparticles were analyzed by dynamic light scattering (DLS) and electrophoretic mobility, respectively, using a Nano-ZS (Malvern Instruments, Malvern, UK).

The morphology of particles was examined by Atomic Force Microscopy using a Nanoscope IV Bio-scopeTM (Veeco Instruments, Santa Barbara, CA, USA). Imaging was done using taping mode and a silicon cantilever with a spring constant of approximately 40 N/m and a resonance frequency of about 170 kHz. The scan speed applied was 0.2 Hz.

2-3. General procedure to produce cyclodextrin macromolecules

β-CD (3.41 g, 3 mmol) was dissolved in 50 mL anhydrous 1-methyl-2-pyrrolidione (NMP) and was cooled to 0 °C for 2 h. Reagent(acid chloride, 200 mmol) dissolved in anhydrous NMP (50 mL) at room temperature was then added drop wise to the β-CD solution with magnetic stirring (200-300 rpm). The reaction temperature was maintained at 0 °C for 2 h and then raised slowly to 40 °C. The reaction was continued for 6 hours at 40°C and subsequently by room temperature overnight. Macromolecule was washed first three times with water/ THF by reprecipitation and subsequently three times with water/CH_2Cl_2 by separating funnel. The organic layer was evaporated. Drying over night in vacuumed box followed to completely remove residual water**.** Degree of substitution was calculated by 1H NMR.

2-3-1. Heptakis[2,3,6-tri-O-(cyclohexyl)]-b**-cyclodextrin (21-Cyclo-CD).**

Degree of substitution: 3.0

Yield of macromonomer: 31.9 %, 1.51 g.

^{1}H NMR ($CDCl_3$, 400 MHz): 1.2-2.5(33H, Cyclohexane unit), 3.5–5.4 (7H, sugar residues)

2-3-2. Heptakis[2,3,6-tri-O-(phenyl alkyl)]-b**-cyclodextrin (21-Phenyl-CD).**

Degree of substitution: 3.0

Yield of macromonomer: 21.3 %, 1.02 g.

^{1}H NMR ($CDCl_3$, 400 MHz): 3.5(s, 6H, CH_2 alkyl group), 3.8–5.4 (7H, sugar residues), 7.0-7.2(m, 15H, phenyl group)

2-3-3. Heptakis[2,3,6-tri-O-(2-bromo-2-methylpropionyl]-b-cyclodextrin (21-Br-CD)

Macromoleule was characterized following the procedure described by Ohno and coauthors[10, 11].

Degree of substitution: 3.0

Yield of macromonomer: 64.51 %, 8.21 g.

^{1}H NMR ($CDCl_3$, 400 MHz): 1.9(s broad, 126H, CH3), 3.5–5.4 (49H, sugar residues)

^{13}C NMR ($CDCl_3$, 400 MHz): 30.8–31.3 (*a*-CH3), 56.4 (*C*Br), 64.3-97.7 (sugar carbons), 171.4 (C=O).

2-4. Preparation of nanoparticles

The respective polymer was dissolved in ethyl acetate (1 mg/mL). This organic solution was dropped to 4 mL aqueous PVA solution (1% w/v). This biphasic system was emulsified with a high speed homogenizer (Ultra Turrax® Ika®, Brasil Ltda, Taquara, Brasil) at 14000 rpm for 10 minutes. Then, 5 mL ultra purified water (MilliQ water) was added to ensure complete diffusion of the organic solvent to the aqueous phase. Finally, the organic solvent was evaporated by stirring in an open vessel in the fume hood overnight at room temperature. The obtained nanoparticle dispersions were filled up to the end volume of 10 mL by addition of purified water. Characterization of the nanoparticle preparations was done by DLS and AFM as described above. Results are means of at least 3 measurements.

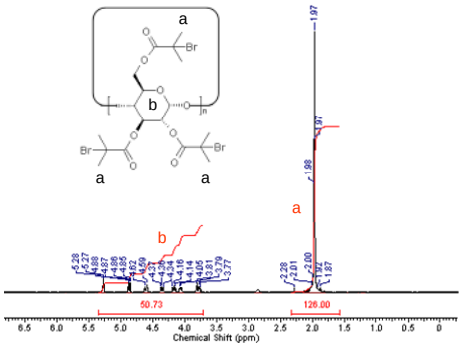
3. Results and Discussion

As depicted in Scheme 1, 3 different kinds of modified cyclodextrins were produced. 21-cyclo-CD and 21-phenyl-CD were synthesized in order to produce a highly hydrophobic modified CD. Aromatic groups of 21-phenyl-CD expected to increase the solubility of actives to the nanoparticles. 21-bromo-CD was produced as an intermediate of star polymer[10, 11, 15-17].

3-1. Characterization of CD macromolecules

a) NMR Spectra of 2-Br-CD

(β-CD modified with 2-bromo-2-propanoyl bromide)



b) IR Spectrum of native β-CD and 21-Br-CD

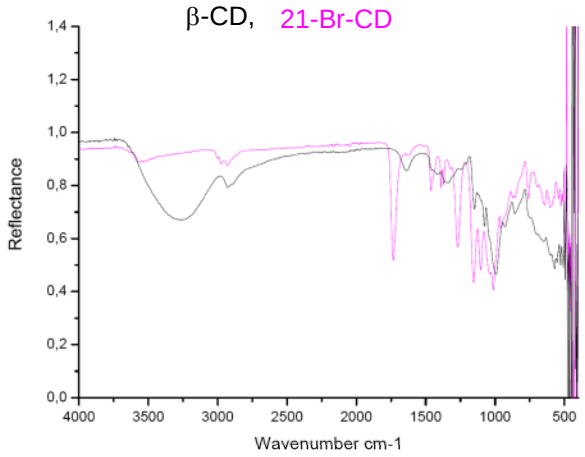


Figure1. Characterization of 21-Br-CD a)1H NMR in $CDCl_2$ b) IR

Figure 1 show the IR (1-a) and NMR (1-b) spectrum of characterization of synthesized 21-Br-CD. The broad peak observed around 3500cm^{-1} attribute to –OH group of Cyclodextrin (black color line). This peak disappeared and a new peak around 1750cm^{-1}, which attribute to ester-C=O- group, was detected after the esterification reaction. It indicated that all OH groups were reacted with acid halide. In addition NMR-integration also proof that all OH groups were replaced by 2-Bromoisobutylic group.

In order to be used for nanoparticle formation by emulsion-diffusion technique, the polymers have to be soluble in organic solvents. Hence the solubility of the CD based macromolecules in various solvents was examined[18].

Modified with	**Code**	**Water**	**Ethyl acetate**	**THF**	**Toluene**
-	β-CD	+	-	-	-
Cyclohexanecarbonyl chloride	21-Cyclo-CD	-	+	+	+
Phenylacetyl chloride	21-Phemyl-CD	-	+	+	+
2-bromo-2-methylpropyl chloride	21-Br-CD	-	+	+	+

+; More than 0.1% w/v soluble in solvent , -; Not soluble

Table 1. Solubility of modified Cyclodextrins.

Table 1 shows the solubility of modified cyclodextrins. 1mg polymer was dissolved in 1ml different organic solvent and stirred 1 hour by room temperature. All macromolecules were not soluble in water but soluble in unpolar organic solvent such as toluene and ethylacetate. These results also indicate that chemical modification of CD worked successfully.

3-2. Characterization of Nanoparticle formulations

The hydrodynamic mean diameter of the NP formulations was determined using intensity weighted analysis of DLS measurement. Table 2 shows the particle size and polydisprsity index (PDI) of produced cyclodextrin nanoparticles.

Modified with	Code	Particle Size(nm)	PDI
-	β-CD	-	-
Cyclohexanecarbonyl chloride	21-Cyclo-CD	163.5 ± 2.63	0.06 ± 0.02
Phenylacetyl chloride	21-Phemyl- CD	166.6 ± 3.9	0.069 ± 0.029
2-bromo-2-methylpropyl chloride	21-Br-CD	190.1 ± 0.75	0.077 ± 0.023

Table 2. Comparison of NP formulations made from the synthesized hydrophobic CD with regard to particle size and particle size distribution as determined by DLS.(Mean ± SD, n=3)

All cyclodextrin macromolecules produced well size controlled nanoparticles, whose particle sizes were around 170.0 nm and small PDIs (<0.1). It is due to the well controlled molecular weight of cyclodextrin macromolecules compared to the natural polymer starch that modified cyclodextrin enable the formation of nanoparticles dispersions with a narrow size distribution as was indicated by the low PDI values. Nanoparticles made from hydrophobical modified starch using the same technology showed comparative broader PDI (around 0.15), most probably due to its more inhomogeniouse molecular weight[19].

3-3. Storage stability of NP formulations

To keep a stability of nano particles in water in a certain period of time is also important factor from commercial points of view. Therefore stability test of NP with 21-cyclo-CDs macromoleclue at 4°C in 6 weeks was examined. Particle size was analyzed immediately after preparation and subsequently after 3 and 6 weeks of storage.

	Nanoparticle		**after 3 weeks**		**After 6 weeks**	
Code	**Size(nm)**	**PDI**	**Size(nm)**	**PDI**	**Size(nm)**	**PDI**
21-Cyclo-CD	163.5 ±2.63	0.06 ±0.02	169.9 ±1.78	0.066 ±0.007	167.1 ±1.05	0.059 ±0.023

Table 3. Storage stability of 21-Cyclo-CD NP in aqueous dispersion. Particle size was monitored over 6 weeks storage at 4°C(Mean ± SD, n=3).

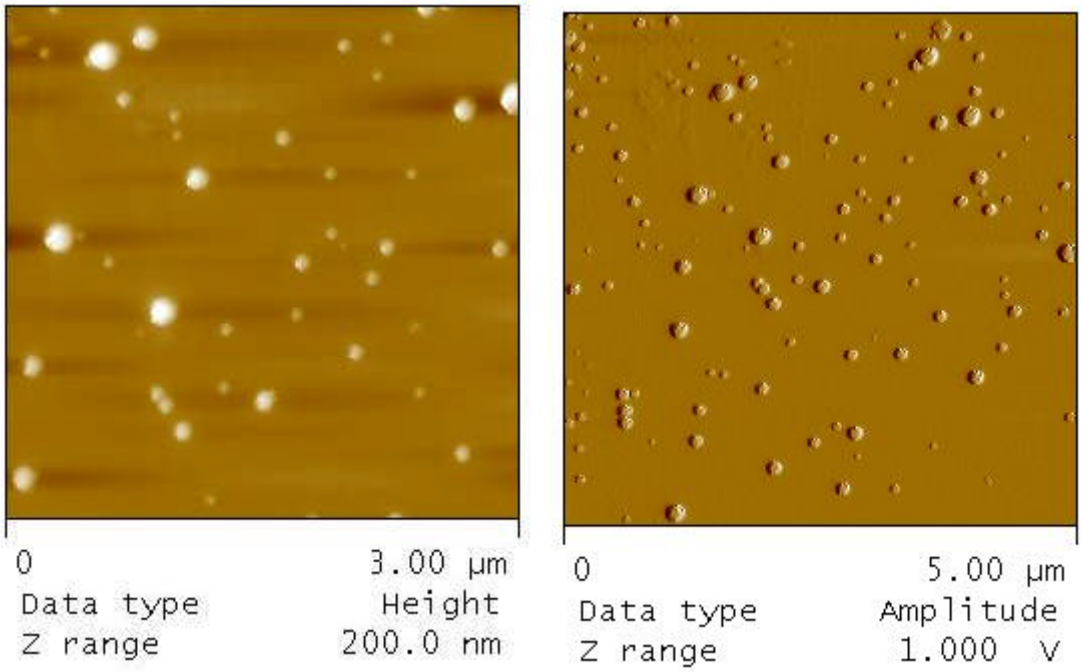
Table 3 summarizes the results of particle size measurements. NP keeps well controlled particle size distribution (PDI<0.1) and particle size (around 170 nm), even after 6week storage. This result also indicates that NP with 21-Cyclo-CD has commercial acceptable storage time.

3-4. Shape and Morphology of NP formulations

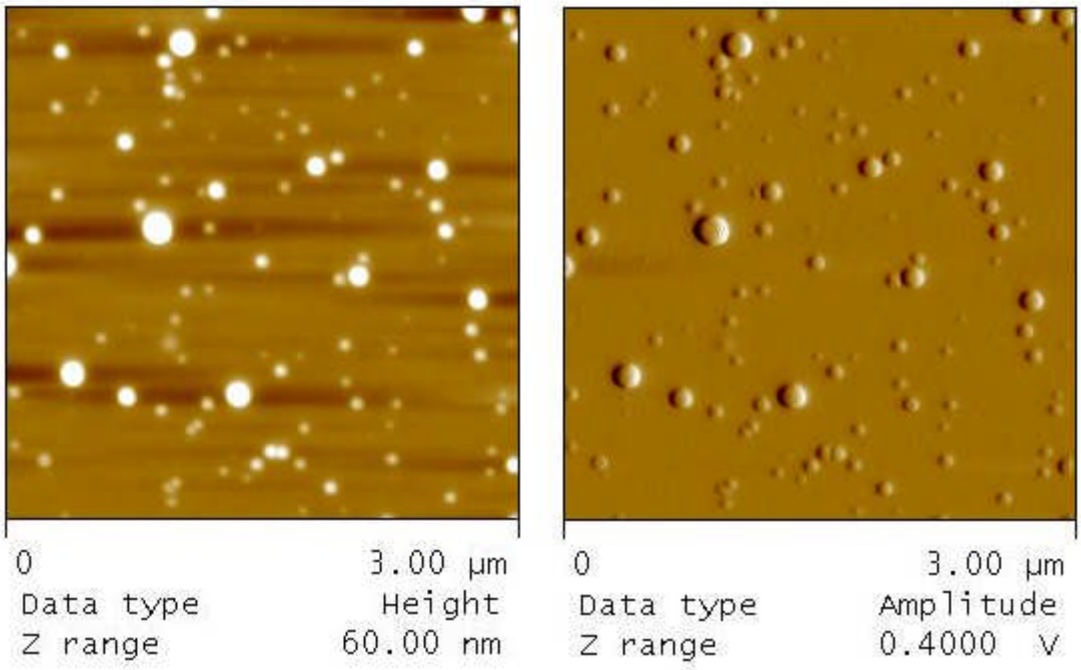
The three different CD-based nanoparticle formulations were imaged by AFM (Figure2-a,b,c,d) in order to get a better knowledge of their morphology and surface. In all cases the particles were spherical shaped and had a smooth surface. The AFM pictures confirmed in principle the size determined by DLS. However, they also revealed the presence of a population of smaller particles which could not been detected by dynamic light scattering.

Figure 2. AFM photos of NPs showing height image (left) and amplitude image (right)

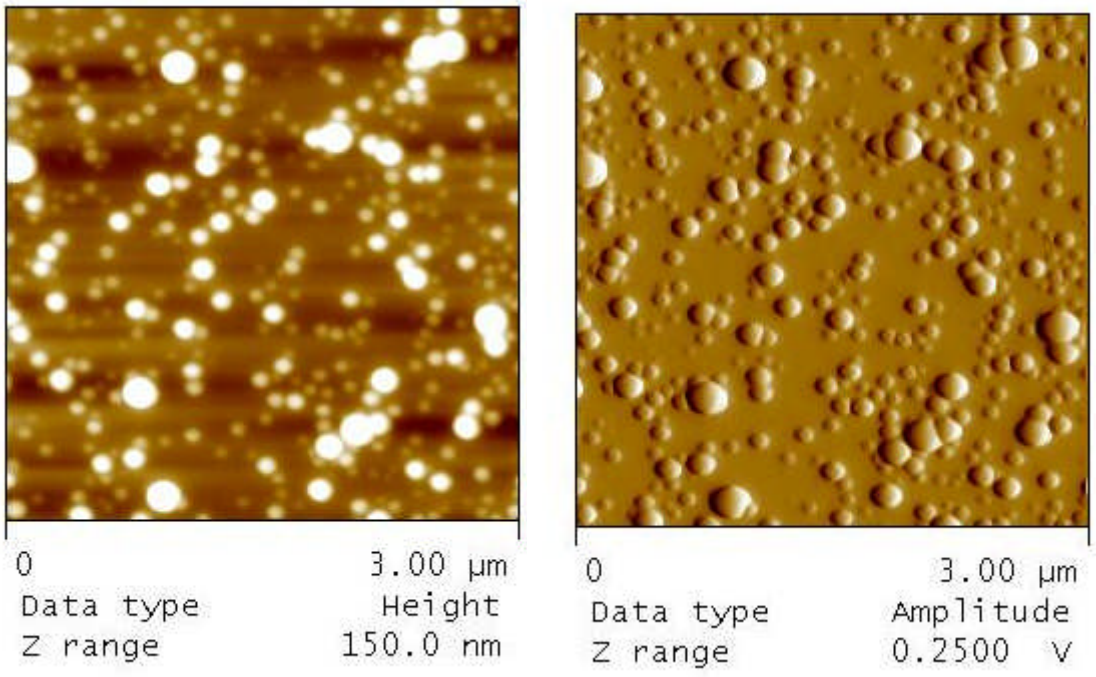
-a)21-Cyclo-CD



-b)21-Phenyl-CD



-c)21-Br-CD



4. Conclusion

The new synthesized hydrophobic modified β-CDs represented a promising platform in the area of nanoparticulate carriers.

5. Acknowledgement

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VII Summary

The present thesis describes the synthesis and characterization of graft copolymers from carbohydrates and formulation of nanoparticles for drug carrier applications. Side chains were produced starting from carbohydrate based macroinitiators via Atom Transfer Radical Polymerization (ATRP). Hydrophobically modified carbohydrate derivatives have a potential to serve as a new platform for biodegradable and biocompatible nanocarriers.

1.β-CD

The conclusion of this study was to synthesize hydrophobically modified cyclodextrins as a new platform for biocompatible and biodegradable nano-carriers. Star shaped polymers with a β-Cyclodextrin (β-CD) core and hydrophobic arms composed of methyl methacrylate (MMA) and tert-butyl acrylate (t-BA) were produced. The synthesis was realized by atom transfer radical polymerization (ATRP) using Nickel as a catalyst. The Ni^{2+} catalyst complexed with a hydrophilic phosphine such as tris(4-methoxyphenyl)phosphine made it possible to accelerate the polymerization. Using this system, a monomer conversion of more than 80% was achieved in optimized reaction conditions. Moreover, the Ni catalysts facilitated its removal by simple extraction with water down to a residual content of 30ppm.

Nanoparticles (NPs) were formed from the CD graft polymers by the emulsion-diffusion technique using poly vinyl alcohol as the dispersant. Resulting NPs, which show high stability during 6 weeks of storage (see Table 1), were uniform and round shaped and showed particle sizes below 200 nm (see Figure1), and narrow size distributions (PDI<0.1). The MMA and t-BA arms are expected to increase the encapsulation of active substances into the NPs and allow the controlled drug release of them. These new CD-star polymers provide a promising platform for preparing nanoparticulate drug delivery systems due to an improvement of biocompatibility by more efficient and easier product purification. Additionally, cell viabilities remain unaffected by these nanoparticles, they are good candidates for the delivery of drugs.

Table 1. Stability of NPs of 3 different β CD-star polymers

Run	Arm chain of β-CD starpolymer	Length of the arm	Mw/Mn	Nanoparticle size(nm)	PDI	After 3 weeks size(nm)	PDI	After 6 weeks size(nm)	PDI
1	MMA-tBA copolymer	20 (10 MMA 10 tBA)	1.6	190.1	0.077	191.5	0.084	188.2	0.078
2	MMA-tBA copolymer	20 (10 MMA 10 tBA)	1.06	177	0.059	181.2	0.064	179.5	0.062
3	MMA Homopolymer	50	1.58	184.8	0.086	189.2	0.049	184.4	0.075

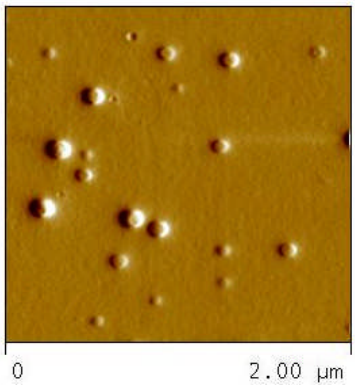


Figure 1. Morphology of nanoparticles from β-CD-star polymer with MMA and tBA by AFM using amplitude mode.

2. Starch

Graft polymers from an enzymatic decomposed starch as the main chain, with controlled molecular weight side chains were produced by ATRP. Polymerization of MMA proceeded in the side chain and more than 70% of the monomer was consumed in this system. These graft polymers were hydrophobic enough to form nanoparticles by the emulsion diffusion technique. The MMA-arm was expected to increase the encapsulation of actives into the nanoparticles (NPs) and allow controlled drug release. NPs prepared by the emulsion-diffusion technique can be characterized by a small size (<200 nm), uniform shape and narrow size distribution (PDI=0.12-0.18). Since cell viabilities remain unaffected by these nanoparticles, they are good candidates for the delivery of drugs (Figure 2).

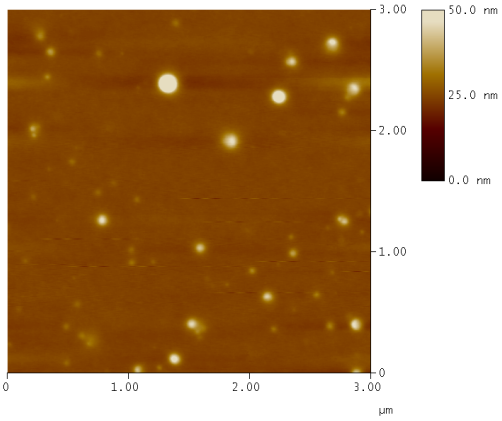


Figure 2. AFM pictures of nanoparticles of starch-PMMA graft polymer using amplitude mode

VIII. Appendix

1.Patent EP 09151043.8

This study was patented by the authors with following claims under special advice of C.Wolf(Patent lawyer, BASF).

1. Copolymer comprising starch as a backbone molecule, a covalently bound linker and a polymerized chain of monomers comprising at least one olefin group bound to the linker, wherein the degree of substitution of hydroxy-groups of the backbone is in the range of 0.05 to 3, preferably 0.1-2, more preferably 0.15-1.5, particularly 0.2-1 and most preferred 0.3-0.5.

2. Copolymer according to claim 1, wherein copolymer is a graft polymer.

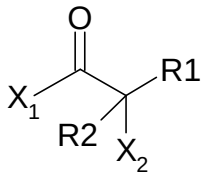
3. Copolymer according to claim 1 or 2, wherein the starch is native starch or physically modified starch, preferably hydrolyzed, particularly enzyme hydrolyzed, native or physically modified starch.

4. Copolymer according to claim 3, wherein the hydrolyzed native starch has a MW of from 800 to 500,000 Da, preferably 4,000 to 100,000 Da.

5. Copolymer according to claim 3, wherein the hydrolyzed native starch has a bimodal MW distribution with a first maximum of from 800 to 1,000 Da, preferably 1,000 to 2,000 Da, and a second maximum of from 5,000 to 100,000 Da, preferably 10,000 to 50,000 Da.

6. Copolymer according to any one of the preceding claims, wherein the starch is water soluble at a degree of at least 30 %w/w at 25 °C.

7. Copolymer according to any one of the preceding claims, wherein the linker is a carbohydrate di-halide, preferably a carboxylic acid di-halide, particularly of the general structure



, wherein R1= H or CH_3, R2 = C1-C6 linear, branched or cyclic alkyl,

preferably R1 and R2 = CH_3,
X_1 and X_2 are independently F, Cl, Br or I, preferably Br or Cl,
and particularly R1 and R2 = CH_3 and X1 and X2 = Br.

8. Copolymer according to any one of the preceding claims, wherein the monomer further comprises an amido- and/or an ester-group adjacent to the olefin group.

9. Copolymer according to any one of the preceding claims, wherein the monomer is an acrylate and/or acryl amide, preferably an alkyl-, fluoroalkyl-, hydroxyalkyl-, aminoalkyl- or N,N-dialkylaminoalkyl-acrylate and/or –acrylamide, particularly at least one monomer selected from the group consisting of methylacrylate, methacrylate, methylmethacrylat (MMA), Dimethylaminoethylmethacrylate (DMAEMA), hydroxyethylmethacrylate (HEMA), hydroxyethylacrylate (HEA), tertiar-butylacrylate (tBA), N-isopropylacrylamide (NIPAM) and methacrylamide, most preferably tertiar-butylacrylat.

10. Copolymer according to any one of the preceding claims, wherein the Monomer is styrene or a styrene derivative, preferably alkyloxy styrene, particularly methoxystyrene.

11. Copolymer according to any one of claims 1-4 or 6-10, wherein the MW of the copolymer is 4,500 – 5,000,000 Da, preferably 5,000 – 1,000,000 Da.

12. Copolymer according to any one of claims 1-3 or 5-10, wherein the MW of the copolymer has a bimodal MW distribution with a first maximum of from 2,000 to 800,000 Da and a second maximum of from 14,000 to 15,000,000 Da.

13. Copolymer according to any one of the preceding claims, wherein the length of the polymer chain of monomers is 10-150, preferably 50-120, particularly ca. 100.

14. Copolymer according to any one of the preceding claims, wherein the polymer is obtained by an atom transfer radical polymerization (ATRP) reaction.

15. Method of preparing a copolymer comprising the steps of reacting starch as a backbone molecule with a linker to give a macro initiator and reacting the macro initiator with monomers comprising at least one olefin-group in the presence of a nickel(II) catalyst via an ATRP reaction.

16. Method according to claim 15, wherein the copolymer is a graft polymer.

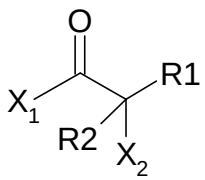
17. Method according to claim 15, wherein preferably native or physically modified starch is hydrolyzed, preferably by at least one enzyme, particularly by at least one of the group consisting of amylase, iso-amylase and alpha-amylase, most preferably by iso-amylase and alpha-amylase, before reacting with the linker.

18. Method according to claim 15, wherein the MW of the hydrolyzed starch is from 800 to 500,000 Da, preferably 4,000 to 100,000 Da.

19. Method according to claim 15, wherein the hydrolyzed native starch has a bimodal MW distribution with a first maximum of from 800 to 1,000 Da, preferably 1,000 to 2,000 Da, and a second maximum of from 5,000 to 100,000 Da, preferably 10,000 to 50,000 Da.

20. Method according to claim 15, wherein the starch is water soluble at a degree of at least 30 %(w/w) at 25 °C.

21. Method according to claim 15, wherein the linker is a carbohydrate di-halide, preferably a carboxylic acid di-halide, particularly of the general structure



, wherein R1= H or CH_3, R2 = C1-C6 linear, branched or cyclic alkyl,

preferably R1 and R2 = CH_3,
X1 and X2 are independently F, Cl, Br or I, preferably Br or Cl,
and particularly R1 and R2 = CH_3 and X1 and X2 = Br.

22. Method according to claim 15, wherein the monomer further comprises an amido- and/or an ester-group adjacent to the olefin group.

23. Method according to claim 15, wherein the monomer is an acrylate and /or acryl amide, preferably an alkyl-, fluoroalkyl-, hydroxyalkyl-, aminoalkyl- or N,N-dialkylaminoalkyl-acrylate and/or –acrylamide, particularly at least one monomer selected from the group consisting of methylacrylate, methacrylate, methylmethacrylate (MMA), Dimethylaminoethylmethacrylate (DMAEMA), hydroxyethylmethacrylate (HEMA), hydroxyethylacrylate (HEA), tertiar-butylacrylate (tBA), N-isopropylacrylamide (NIPAM) and methacrylamide, most preferably tertiar-butylacrylate (tBA).

24. Method according to claim 15, wherein the Monomer is styrene or a styrene derivative, preferably alkyloxy styrene, particularly methoxystyrene.

25. Method according to claim 15, wherein the Ni-catalyst has phosphine, preferably tris-phenylphosphine, particularly tris-methoxyphenylphosphine, ligands.

26. Method according to claim 15, wherein the Ni-catalyst is one selected from the group consisting of Ni Br_2(tris-phenylphosphine)$_2$,
Ni Br_2(tris(4-methoxyphenyl)phosphine)$_2$,
Ni Br_2(tris(ortho-methoxyphenyl)phosphine)$_2$,
Ni Br_2(tris(meta-methoxyphenyl)phosphine)$_2$,
Ni Br_2(tris(ortho-tolyl)phosphine)$_2$,
Ni Br_2(tris(meta-tolyl)phosphine)$_2$ and
Ni Br_2(tris(para-tolyl)phosphine)$_2$,
preferably Ni Br_2(tris(4-methoxyphenyl)phosphine)$_2$

27. Method according to claim 26, wherein instead of Br another halide selected from the group consisting of Cl, F and I is present in the Ni catalyst.

28. Method according to claim 15, wherein the Ni-catalyst is formed in situ by ligand exchange of phosphine ligands, preferably tris-phenylphosphine ligands are changed for phosphine ligands with more electrons, preferably one of the group consisting of
tris(4-methoxyphenyl)phosphine,
tris(ortho-methoxyphenyl)phosphine,
tris(meta-methoxyphenyl)phosphine,
tris(ortho-tolyl)phosphine,
tris(meta-tolyl)phosphine and
tris(para-tolyl)phosphine,
particularly tris(4-methoxyphenyl)phosphine.

29. Method according to claim 15, wherein the reaction is carried out at 25-80 °C, preferably at 60-80 °C.

30. Method according to claim 15, wherein the reaction is carried out in a polar organic solvent, preferably in one selected from the group consisting of DMF, DMAc, NMP, DMSO, THF, AcCN, Acetone, ethyl acetate and mixtures thereof or with one of the group of non-polar solvents consisting of hexane, toluol, cyclohexane and benzene in a ration of 5-10:1, particularly in DMSO.

31. Method according to claim 15, wherein the reaction is carried out under water free conditions

32. Method according to claim 15, wherein the reaction is carried out under non-oxygen conditions, preferably under nitrogen or argon.

33. Copolymer obtained by a method according to any one of claims 15-32.

34. Copolymer according to Claim 33, wherein the copolymer is a graft polymer.

35. Nanoparticles comprising at least one starch derivative, wherein the starch derivative is a copolymer according to Claim 1 or 33.

36. Nanoparticles according to claim 35 wherein the nanoparticles have an average particle size diameter in the range of 20 to 500 nm.

37. Method of preparing nanoparticles containing at least one starch derivative copolymer according to Claim 1 or 33 comprising the step of preparing an emulsion which contains an aqueous phase comprising an emulsifier and an organic phase comprising an organic solvent and the starch derivative copolymer.

38. Method according to claim 37 wherein the said emulsion is an oil/water emulsion comprising alkyl acetate as organic solvent.

39. Method according to one of the claims 37 or 38 wherein the said emulsion comprises ethyl acetate or acetone as organic solvent and the starch derivative copolymer in an amount from 0.05 to 2 % weight/volume (w/v).

40. Method according to one of the claims 37 to 39 wherein the emulsifier in the aqueous phase is added to the aqueous phase in an amount from 0.01 to 2 % w/v of the aqueous phase.

41. Composition comprising at least one active ingredient contained in nanoparticles according to claim 35.

42. Composition according to claim 41 wherein said active ingredient is encapsulated in the nanoparticles.

43. Composition according to claim 41 or 42 wherein said active ingredient is a pharmaceutical ingredient.

44. Composition according to any one of claims 41 to 43 wherein said active ingredient is select from the group consisting of hormones, alkaloids, non-steroidal anti-inflammatory drugs.

45. The use of nanoparticles according to claim 35 for the preparation of pharmaceutical, cosmetic or food compositions.

46. The use according to claim 45, wherein the pharmaceutical composition is a drug delivery system (DDS), preferably a transdermal drug delivery system (TDDS) or oral drug delivery system (ODS).

2.Patent EP 09151010.7

This study was patented by the authors and T.Stauner with following claims under special advice of C.Wolf(Patent lawyer, BASF).

Claims

1. Nanoparticles comprising at least one starch derivate, wherein the starch derivative is a hydrophobic starch derivative with an average degree of substitution of the hydroxyl-groups (D_s) in the range of 0.5 to 2.75.

2. Nanoparticles according to claim 1 wherein the hydrophobic starch derivatives is selected from the group consisting of starch derivatives with hydrocarbon side chains.

3. Nanoparticles according to one of the claims 1 or 2 wherein the hydrophobic starch derivative is selected from the group consisting of C_1-C_6 alkyl starch derivatives.

4. Nanoparticles according to one of the claims 1 to 3 wherein the nanoparticles have an average particle size diameter in the range of 20 to 500 nm.

5. Nanoparticles according to one of the claims 1 to 4 wherein the starch derivative is prepared from starch selected from the group of native starch, enzymatic modified starch, acid modified starch or mechanically modified starch.

6. Method of preparing nanoparticles containing at least one starch derivative with an average degree of substitution of the hydroxyl-groups (D_s) in the range of 0.5 to 2.75 comprising the step of preparing an emulsion which contains an aqueous phase comprising an emulsifier and an organic phase comprising an organic solvent and a hydrophobic starch derivative.

7. Method according to claim 6 wherein the said emulsion is an oil/water emulsion comprising alkyl acetate as organic solvent.

8. Method according to one of the claims 6 or 7 wherein the said emulsion comprises ethyl acetate as organic solvent and as hydrophobic starch derivative an alkyl starch derivative in an amount from 0.5 to 2 % weight/volume (w/v).

9. Method according to one of the claims 6 to 8 wherein the emulsifier in the aqueous phase is added to the aqueous phase in an amount from 0.01 to 2 % w/v of the aqueous phase.

10. Composition comprising at least one active ingredient contained in nanoparticles comprising starch derivatives according to one of the claims 1 to 5.

11. Composition according to claim 10 wherein said active ingredient is encapsulated in the nanoparticles.

12. Composition according to one of the claim 10 or 11 wherein said active ingredient is a pharmaceutical ingredient.

13. Composition according to one of claim 10 to 12 wherein said active ingredient is select from the group consisting of hormones, alkaloids, non-steroidal anti-inflammatory drugs.

14. The use of nanoparticles according to one of the claims 1 to 5 for the preparation of pharmaceutical, cosmetic or food compositions.

15. The use according to claim 14, wherein the pharmaceutical composition is a transdermal drug delivery system (TDDS).