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DIPLOMARBEIT

Interaction of bioadhesive nanoparticles with an artificial intestinal epithelium

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Verfasserin:	Adina-Katharina Ilyes
Matrikel-Nummer:	0202877
Studienrichtung /Studienzweig (lt. Studienblatt):	A 449 Pharmazie
Betreuerin / Betreuer:	Ao. Univ.-Prof. Mag. Dr. Michael Wirth

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Der letzte Schritt der Vernunft ist anzuerkennen,

dass es unendlich viele Dinge gibt, die über sie hinausgehen.

Sie ist nur schwach, wenn sie nicht so weit geht, das anzuerkennen.

Wenn die natürlichen Dinge schon über sie hinausgehen,

was wird man dann von den übernatürlichen sagen?

Pensée Nr.188, Blaise Pascal

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INHALTSVERZEICHNIS

PROBLEMSTELLUNG

Eines der Kerngebiete der pharmazeutisch-technologischen Forschung stellt die Entwicklung von Formulierungen für biotechnologisch hergestellte Arzneistoffe dar. Diese Klasse von Wirkstoffen wird als äußerst zukunftsträchtig angesehen und umfasst unter anderen Desoxyribonukleinsäuren (DNS), Ribonukleinsäuren (RNS), Peptide und Proteine. Der hohen Wirksamkeit dieser Makromoleküle steht als gravierender Nachteil ihre zumeist unzureichende Stabilität nach Applikation *in vivo* gegenüber. Ein vielversprechender Ansatz um diese Substanzen sowohl vor dem Abbau durch Enzyme als auch vor der Denaturierung in sauren Kompartimenten zu schützen, ist die Verkapselung in Nano- und Mikropartikeln. Um als Arzneistoffträger angewandt werden zu können, müssen die Teilchen aus biokompatiblen und bioabbaubaren Polymeren bestehen. Zu diesem Zweck steht eine Vielzahl von natürlichen und synthetischen Polymeren zur Verfügung, die den inkorporierten Wirkstoff verzögert freisetzen.[1,2] Innerhalb dieser Gruppe von Polymeren werden die Homo- und Copolymere der α-Hydroxysäuren Milch- und Glykolsäure (Poly(D,L-lactic acid) (PLA), Poly(D,L-lactic-co-glycolic acid) (PLGA)) besonders häufig verwendet.[3,4] Die Gründe dafür liegen sowohl in der Zulassung von PLGA als Hilfsstoff durch die Food and Drug Administration (FDA) als auch in der breiten kommerziellen Verfügbarkeit des Polymers.

Neben dem Schutz des Arzneistoffs eröffnet die Verwendung von partikulären Trägersystemen auch Möglichkeiten zur Steuerung der Biodistribution. Beispielsweise kann durch Modifikation der Partikeloberfläche mit „targeter“-Molekülen eine bevorzugte Anreicherung des Wirkstoffs im erkrankten Gewebe erzielt werden. So könnte der sonst üblichen „Überschwemmung“ des Organismus mit hochwirksamem Arzneistoff entgegengewirkt und dadurch der Schweregrad und die Häufigkeit des Auftretens von Nebenwirkungen reduziert werden.

Ein weiterer Vorteil von Nanopartikeln ist deren theoretisches Potential zur Translokation über Epithel- und Endothelbarrieren im Körper. Aus biopharmazeutischer Sicht ist diese Eigenschaft besonders bei der peroralen Verabreichung von Arzneistoffen von Interesse, da dieser Weg

nach wie vor die einfachste und beliebteste Methode der Arzneimitteleinnahme darstellt. Hierbei könnten Nanopartikel als schützendes Trägersystem fungieren, welches über das gastrointestinale Epithel resorbiert wird und schlussendlich den Wirkstoff im Blut- und Lymphsystem freisetzt. Untersuchungen mit Kolloiden aus Polystyren (PS) haben gezeigt, dass dieser Resorptionsweg tatsächlich die Aufnahme von Nanopartikeln in die systemische Zirkulation erlaubt.[5] Laut ersten Studien ist das Ausmaß der Resorption aber vermutlich zu gering um einen therapeutisch effektiven Plasmaspiegel zu erzielen.[6] Ein Hauptgrund dafür könnte in der geringen Bioadhäsivität der eingesetzten Polymerteilchen liegen.[7] Diese Eigenschaft wurde jedoch nicht nur für PS sondern auch für das biokompatible und bioabbaubare PLGA beschrieben.[8,9] Um die Anreicherung von PLGA-Nanopartikeln (PLGA-NP) am Epithel zu fördern, können die Partikel mit mukoadhäsiven Polymeren wie Chitosan überzogen werden.[10] Eine weitere Möglichkeit um die Bioadhäsivität von Teilchen zu verbessern stellt die Konjugation mit Lektinen dar. Lektine sind Proteine bzw. Glykoproteine, die mit der Glykokalyx der Plasmamembran eukaryotischer Zellen interagieren können. Jedes Lektin ist durch seine jeweils spezifische Wechselwirkung mit einer bestimmten Zuckerstruktur charakterisiert. Durch ein screening von Lektinen mit unterschiedlichen Zuckerspezifitäten kann das Protein identifiziert werden, das für die jeweilige Zellart die stärkste Bioadhäsion aufweist. Für intestinale Epithelzellen wurde eine hohe Bindung von Weizenlektin (WGA) beschrieben. WGA ist ein säurestabiles, dimeres Protein mit vier Bindungsstellen zur Wechselwirkung mit N-Acetyl-D-Glucosamin- und N-Acetylneuraminsäureresten.[11,12] Nach Bindung an die Membran von intestinalen Epithelzellen wird ein Großteil der assoziierten WGA-Moleküle internalisiert.[13] Um diese vorteilhaften cytoadhäsiven aber auch cytoinvasiven Eigenschaften auf Polymerteilchen zu übertragen, kann WGA kovalent an der Partikeloberfläche immobilisiert werden. Bei Partikeln aus PLGA stehen zu diesem Zweck die freien endständigen Carboxylgruppen des Polymers zur Verfügung. Diese reagieren nach Aktivierung mit N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimid Hydrochlorid (EDAC) und N-Hydroxysuccinimid (NHS) mit den Amingruppen der Proteine zu Amiden. Derart modifizierte WGA-Partikel zeigen verglichen mit unmodifizierten Teilchen eine deutlich erhöhte Bindung an

epitheliale Caco-2 Einzelzellen.[8] Aus biopharmazeutischer Sicht ist die Aussagekraft von Einzelzellversuchen jedoch limitiert, da kaum Rückschlüsse auf die Interaktion von Partikeln mit einem Zellgewebe gezogen werden können. Dazu eignen sich Zellmonolayer deutlich besser. Ein pharmazeutisch akzeptiertes *in vitro* Modell stellen in diesem Zusammenhang polarisierte Caco-2 Monolayer dar, die sowohl morphologisch als auch funktionell weitgehend dem humanen Dünndarmepithel entsprechen.[14]

Im Rahmen der vorliegenden Diplomarbeit sollte untersucht werden, inwieweit die Interaktion von biokompatiblen und biodegradierbaren PLGA-Nanopartikeln mit artifiziellem humanem Dünndarmgewebe durch Konjugation mit WGA verbessert werden kann. Um die Zahl der zellgebundenen Partikel erfassen zu können, sollen PLGA-NP mit dem Fluoreszenzfarbstoff BODIPY® 493/503 (BOD; 4,4-Difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-5-indazen) markiert werden.[8] Nach Charakterisierung von Kontroll- und bioadhäsiven Partikeln hinsichtlich ihrer Stabilität in ausgewählten Puffern und Medien, sollen Partikelbindungsstudien mit Caco-2 Zellmonolayern in Mikrotiterplatten durchgeführt und das Ausmaß der Interaktion sowohl fluorimetrisch als auch fluoreszenzmikroskopisch analysiert werden.

ERGEBNISSE UND DISKUSSION

Im Rahmen der vorliegenden Diplomarbeit konnte zu den Publikationen

- „**Bionanoprobes to study particle-cell interactions**“

  C. Fillafer, D.S. Friedl, A.K. Ilyes, M. Wirth, F. Gabor, *Journal of Nanoscience and Nanotechnology* **2009**, 9, 3239.

- “**Interaction of bioadhesive nanoparticles with an artificial intestinal epithelium**”

  C. Fillafer, A.K. Ilyes, M. Wirth, F. Gabor, wird eingereicht bei *Scientia Pharmaceutica*

(siehe Anhang A und B) maßgeblich beigetragen werden.

Um das Ausmaß der Bindung von Nanopartikeln an Zellen untersuchen zu können, müssen die verwendeten Partikel analytisch erfassbar sein. Zu diesem Zweck werden die Polymerteilchen zumeist mit radioaktiven oder fluoreszierenden Substanzen markiert, wobei fluoreszenzbasierte Techniken aufgrund der unkomplizierten Handhabung und hohen Analysensensitivität bevorzugt werden. In der vorliegenden Arbeit wurde das Fluorophor BOD mittels einer Öl-in-Wasser (o/w) Solvent Evaporation-Technik in PLGA-NP verkapselt.[8] Die im Partikel eingeschlossenen lipophilen Farbstoffmoleküle lagern sich dabei über hydrophobe Wechselwirkungen an den apolaren Polymerketten der Matrix an, werden so verankert und nur geringfügig ins umgebende Medium freigesetzt. (Figure 2, Anhang A) Die mittlere Partikelgröße und der Polydispersitätsindex (PdI) der auf diese Weise hergestellten BOD-markierten PLGA-Nanopartikel (BOD-NP) wurden mittels dynamischer Lichtstreuung bestimmt. Unmittelbar nach der Herstellung waren die BOD-NP durch einen mittleren Durchmesser von 85 nm und eine enge Größenverteilung charakterisiert. Letzteres wird durch den niedrigen Polydispersitätsindex von 0,116 verdeutlicht. (Table 1, Anhang 2) Aufgrund der freien Carboxylgruppen auf der Partikeloberfläche hatten unmodifizierte Nanopartikel ein Zetapotential von -20,4 mV. Im Verlauf des Kopplungsprozesses wurden diese

Carboxylgruppen mit EDAC und NHS aktiviert. Anschließend wurde entweder humanes Serumalbumin (HSA) oder WGA zugesetzt und über Reaktion mit den Aktivestern kovalent an der Partikeloberfläche immobilisiert. Für die nachfolgenden Zellbindungsstudien wurden HSA-BOD-NP als Kontrolle eingesetzt werden, da bekannt ist, dass dieses Protein nur unspezifisch und in sehr geringem Ausmaß an Caco-2 Zellen bindet.

Die Bestimmung der Partikelgrößenverteilung nach der Kopplung ergab für HSA- und WGA-BOD-NP einen mittleren Durchmesser von 98 nm. Sowohl die nur marginale Größenzunahme im Vergleich zu unmodifizierten Nanopartikeln als auch die weitgehend konstanten PdI von 0,127 beziehungsweise 0,120 weisen darauf hin, dass es infolge der Proteinkopplung zu keiner Bildung von Aggregaten kam. Die zwar geringe, jedoch feststellbare Zunahme der mittleren Partikelgröße könnte als indirekter Nachweis für die erfolgreiche Immobilisierung von HSA bzw. WGA an der Teilchenoberfläche dienen. Außerdem wurde im Zuge der Kopplung von Proteinmolekülen an die Partikeloberfläche ein Anstieg des Zetapotentials auf -13,8 mV bei HSA- und -15.2 mV bei WGA-BOD-NP festgestellt. Diese Verringerung des Oberflächenpotentials deutet auf die Derivatisierung von geladenen Carboxyl- zu ungeladenen Amidgruppen hin. Darüber hinaus könnte auch eine Abschirmung unreagierter Oberflächenladungen durch die angelagerten Proteinmoleküle erfolgt sein. Die gemessenen, relativ geringen Oberflächenladungen könnten sich in Bezug auf die Stabilität der Partikelsuspension negativ auswirken. Da bei kolloidalen Systemen mit Zetapotentialen von weniger als ±30 mV keine ausreichende elektrostatische Stabilisierung gegeben ist, könnten sich bei der Langzeitlagerung von WGA- und HSA-modifizierten aber auch unmodifizierten BOD-NP Partikelaggregate bilden. Um diesen Mangel an elektrostatischer Stabilisierung zu kompensieren, wurde während des Herstellungs- und Kopplungsprozesses etwa 1% (w/w) des Tensids Pluronic® F-68 zugesetzt.

Sowohl nach der Herstellung als auch nach der Kopplung mit WGA bzw. HSA liegen BOD-NP in Dispersionsmedien vor, die aufgrund des pH-Werts und osmotischen Drucks nicht

uneingeschränkt für Untersuchungen mit Zellen geeignet sind. Die für die Partikelbindungsstudien verwendeten Caco-2 Zellen sind in dieser Hinsicht zwar vergleichsweise stabil und können auch über längere Zeit in nicht isotonem 20 mM HEPES/NaOH-Puffer pH 7,4 (HEPES) inkubiert werden. Um jedoch Versuche mit anderen Zelllinien über mehrere Stunden durchführen zu können, müssen die Nanopartikel in einem isotonen Medium suspendiert werden. Hierbei kann es zu Agglomeratbildung kommen, da die hohe Ionenstärke von isotonen Lösungen zur Kollabierung des Zetapotentials und folglich zur weitgehenden Beseitigung der interpartikulären elektrostatischen Abstoßung führt. Setzt man Zellkulturmedien ein, kann außerdem der doch beträchtliche Proteingehalt zu einem Quenching der Partikelfluoreszenz führen, wodurch sich Einbußen in der analytischen Sensitivität ergeben. Um ein für weiterführende Studien geeignetes Suspensionsmedium zu identifizieren, wurden unmodifizierte und HSA-modifizierte BOD-NP in isotonem HEPES/NaOH pH 7,4 (isoHEPES), phosphatgepufferter Salzlösung pH 7,4 (PBS), RPMI 1640 Zellkulturmedium (RPMI), einer 0,1% und 1% Lösung von HSA in isoHEPES sowie in destilliertem Wasser als Kontrolle verdünnt. Direkt nach Herstellung der Verdünnung sowie nach Inkubation für 90 min bei 37 °C wurden die Fluoreszenzintensität der Suspension und die mittlere Partikelgröße bestimmt. Wie in Figure 1 (Anhang A) gezeigt, war der mittlere Partikeldurchmesser der unmodifizierten und HSA-modifizierten BOD-NP sowohl unmittelbar nach dem Verdünnen als auch nach Inkubation bei 37 °C in RPMI und allen Puffern konstant. Bezüglich der Fluoreszenzintensität der Partikelsuspension konnten jedoch deutliche Unterschiede zwischen proteinhältigen und proteinfreien Suspensionsmedien festgestellt werden. Während es beim Verdünnen der Partikel mit isoHEPES und PBS zu keiner Änderung der Fluoreszenzintensität im Vergleich zu Wasser kam, konnte in Anwesenheit von Proteinen ein starkes Quenching der Fluoreszenz detektiert werden. Im Fall von HSA hältigem Medium wird das durch eine Reduktion der Fluoreszenzintensität um 36% unmittelbar nach dem Mischen beziehungsweise um 81% nach Inkubation bei 37 °C verdeutlicht. Diese starke Reduktion der Fluoreszenzintensität ist vermutlich auf den Kontakt zwischen Proteinen und BOD an der Partikeloberfläche zurückzuführen. Eine ähnliche Reduktion der

Fluoreszenzintensität von BOD in Proteinlösungen ist bereits in der Literatur beschrieben worden.[15] Um eine entsprechend hohe analytische Sensitivität zu gewährleisten, sollten daher für Zellbindungsstudien mit BOD-NP nur proteinfreie Suspensionsmedien wie PBS oder isoHEPES verwendet werden.

Vor der Charakterisierung der Bindungskinetiken sollte untersucht werden, ob der Differenzierungsgrad von Caco-2 Zelllayern Auswirkungen auf die Bindung von HSA- und WGA-BOD-NP hat. Hierzu wurden Bindungsstudien mit konfluenten Caco-2 Monolayern vier, sechs, acht und elf Tage nach Aussaat der Zellen für 120 min bei 4 °C durchgeführt. Durch Herabsetzen der Inkubationstemperatur auf 4 °C werden sowohl energieabhängige zelluläre Prozesse, wie z.B. die Endocytose, gehemmt als auch die Fluidität des Phospholipidbilayers reduziert. Dadurch soll sichergestellt werden, dass bei den Bindungsstudien tatsächlich nur die Cytoadhäsivität der Partikel erfasst wird. Um beurteilen zu können, ob sich das Ausmaß der N-Acetyl-D-Glucosamin- und N-Acetyl-N-Neuraminsäurereste auf der Zellmembran mit fortschreitender Differenzierung ändert, wurde fluoreszenzmarkiertes WGA (f-WGA) als Kontrolle eingesetzt. Nach Inkubation mit den Analyten wurden die Zelllayer mit HEPES gewaschen, um ungebundene Nanopartikel beziehungsweise freies Lektin zu entfernen. Anschließend wurde fluorimetrisch (Excitation/Emission: 480/525) die zellassoziierte Fluoreszenzintensität als Maß für die Bindung von BOD-NP bzw. f-WGA bestimmt.

Wie in Figure 1 (Anhang B) ersichtlich, blieb die mittlere zellassoziierte Fluoreszenzintensität für f-WGA unabhängig von der Kultivierungsdauer des Zelllayers weitgehend konstant. Dieses Ergebnis deutet darauf hin, dass sich die Anzahl an potentiellen Bindungsstellen für WGA an der Glykokalyx mit dem Alter des Zelllayers nicht verändert. Im Gegensatz dazu konnten bei der Bindung von WGA-modifizierten Nanopartikeln deutliche Unterschiede in Abhängigkeit vom Alter der verwendeten Caco-2 Monolayer festgestellt werden. Während die zellassoziierte Fluoreszenzintensität bis zu einer Kultivierungsdauer von acht Tagen kontinuierlich anstieg,

wurde bei elf Tage alten Zellen eine deutlich reduzierte Bindung beobachtet. Im Fall von HSA-modifizierten Partikeln zeigte sich eine andere Tendenz. Bei vier beziehungsweise sechs Tage alten Zelllayern war die mittlere zellassoziierte Fluoreszenzintensität mit ungefähr 2% der eingesetzten Fluoreszenz erwartungsgemäß gering. Mit zunehmendem Alter der Zellen stieg dieser Wert jedoch auf etwa 5% an. Zusammen mit den Ergebnissen für WGA-BOD-NP lässt sich festhalten, dass der Unterschied zwischen spezifischer und unspezifischer Wechselwirkung bei jüngeren Monolayern stärker ausgeprägt ist. Da die Bindung von f-WGA jedoch weitgehend konstant war, können die beobachteten Variationen jedoch nicht mit Veränderungen in der Zuckerzusammensetzung der Glykokalyx erklärt werden. Im Verlauf der Differenzierung bildet die Zellmembran fingerähnliche Zotten, sogenannte Mikrovilli, die je nach Kultivierungsdauer eine Länge zwischen 0.2 µm und 1.0 µm aufweisen.[16] Dies führt zu einer Vergrößerung der Oberfläche, die in der Folge auch einer unspezifischen Bindung von HSA-BOD-NP vermehrte Möglichkeit zur Anlagerung bietet. Dieser Trend lässt sich auch für WGA-BOD-NP beobachten. Für 11 Tage alte Zelllayer zeigt sich jedoch, dass die nicht spezifische Bindung von HSA-BOD-NP offenbar stärker ansteigt als die spezifische Wechselwirkung von WGA-BOD-NP. Dies könnte darauf zurückzuführen sein, dass die Zugänglichkeit der Kohlenhydratbindungsstellen für die Partikel möglicherweise trotz Oberflächenvergrößerung nicht in gleichem Maße ansteigt. Da im Lauf des Differenzierungsprozess zwischen den Mikrovilli eine Lücke von nur ungefähr 60 nm entsteht, könnte die Bindung von WGA-gekoppelten NP sterisch gehindert sein[17], insbesonders dann, wenn sich die Bindungstellen für WGA bevorzugt nahe der Mikrovilli-Basis befinden. In diesem Fall könnten mit kleineren Nanopartikeln, die in diese Lücken hineindiffundieren können, höhere Bindungsraten erzielt werden.

Für die Partikelbindungskinetiken wurden sechs Tage alte Caco-2 Monolayereingesetzt, wobei die Zelllayer bei 4 °C für bis zu 120 min mit HSA- bzw. WGA-BOD-NP inkubiert und in regelmäßigen Abständen die zellassoziierten Fluoreszenzintensitäten bestimmt wurden. Wie in

Figure 2 (Anhang B) gezeigt, stieg die mittlere zellassoziierte Fluoreszenzintensität mit zunehmender Inkubationsdauer deutlich an. Im Fall von HSA-modifizierten Nanopartikeln entspricht dies einer Zunahme der Bindung von 3.1 ± 0.5% der eingesetzten Partikel nach zehnminütiger Inkubation auf 9.4 ± 0.5% nach einer Inkubationsdauer von 90 Minuten. Bei Verlängerung der Inkubationsdauer für weitere 30 Minuten konnte keine zusätzliche Bindung von HSA-BOD-NP an die Caco-2 Zellen festgestellt werden. Dies deutet darauf hin, dass die Bindungsstellen für eine unspezifische Interaktionen mit albuminmodifizierten Partikeln bereits weitgehend abgesättigt waren. Im Gegensatz dazu konnte für WGA-BOD-NP keine Tendenz einer Sättigung der Partikelbindung beobachtet werden. Während nach zehnminütiger Inkubation mit 1.8 ± 0.3% geringfügig weniger WGA-modifizierte Partikel als HSA-BOD-NP an den Zelllayer gebunden hatten, konnte nach 60-bzw. 120-minütiger Inkubation ein Anstieg auf 10.7 ± 0.9% bzw. 13.9 ± 1.3% detektiert werden. Die Bindung von WGA-BOD-NP an den Zelllayer nach 60-minütiger Inkubation bei 4 °C wurde auch fluoreszenzmikroskopisch erfasst (Figure 4A, Anhang B). Dabei zeigt sich auf den Zellen eine gleichmäßige Verteilung von punktförmigen partikulären Strukturen. Generell lag die Bindung von WGA-BOD-NP an die Caco-2 Zelllayer über die gesamte Versuchszeit im Mittel ungefähr 31,5% höher als bei HSA-BOD-NP. Obwohl die Modifikation mit WGA demnach zu einer im Vergleich mit HSA erhöhten Cytoadhäsion führte, konnten für Monolayer nicht annähernd so hohe Unterschiede in der Bindungsrate festgestellt werden, wie sie für Einzelzellen beschrieben sind.[8] Dies könnte an der unzureichenden Durchmischung des Versuchsansatzes im Mikrotiterplattenwell liegen. Derartige stationäre Inkubationsbedingungen entsprechen auch nicht den am Epithel vorherrschenden Verhältnissen *in vivo*. Durch Simulation einer adäquaten physiologischen Dynamik könnten hydrodynamische Scherkräfte erzeugt werden, die zu einer entsprechenden Reduktion der unspezifischen Wechselwirkung führen sollten. Daher sollte für zukünftige Bindungsstudien eine effektive nicht-invasive Durchmischung der Mikrotiterplattenassays in Betracht gezogen werden. Auf diese Weise könnte einerseits die Aussagekraft des *in vitro* Zellmodells erhöht und andererseits das tatsächliche Ausmaß der unspezifischen Wechselwirkungskomponente bestimmt werden.

Um zu klären, ob membrangebundene HSA- und WGA-BOD-NP in Caco-2 Monolayer aufgenommen werden können, wurde ein pulse-chase Protokoll verfolgt. Dazu wurde die Membran der Zellen im pulse-Schritt 120 min lang bei 4 °C mit Nanopartikeln beladen. Nach Abtrennen der ungebundenen Teilchen folgte ein chase-Inkubationsintervall für 30 beziehungsweise 60 min bei 37 °C. Bei dieser Temperatur ist die Fluidität der Plasmamembran im Vergleich zu 4 °C erhöht und energieabhängige Aufnahmeprozesse können stattfinden. Wird zur Markierung der Nanopartikel ein Fluoreszenzfarbstoff eingesetzt, der nach Internalisation seine Fluoreszenzintensität verändert, sollten auch die cytoinvasiven Eigenschaften der Teilchen bewertet werden können. Im Fall von BOD, das in proteinreichen Vesikeln einen Fluoreszenz-Quench erfährt, kann so eine Partikelaufnahme in Endosomen durch die Abnahme der zellassoziierten Fluoreszenzintensität detektiert werden.[8]

Nach Inkubation der Caco-2 Zellmonolayer für 120 min bei 4 °C mit HSA- bzw. WGA-BOD-NP lag die mittlere zellassoziierte Fluoreszenzintensität bei 92 bzw. 151 Fluoreszenz Einheiten (FU) (Figure 3, Anhang B). Nach Erhöhen der Inkubationstemperatur auf 37 °C kam es sowohl im Fall von HSA- als auch im Fall von WGA-modifizierten Nanopartikeln zu einer Abnahme der zellassoziierten Fluoreszenzintensität. Bei Caco-2 Zelllayern, die mit WGA-BOD-NP beladen wurden, sank die zellgebundene Fluoreszenzintensität verglichen mit dem Startwert innerhalb von 30 min um 44% und nach 60 min um 75%. Diese Abnahme führte auch zu einer Änderung des fluoreszenzmikroskopischen Bildes. Statt der bei 4 °C vorherrschenden gleichmäßig verteilten punktförmigen Fluoreszenz waren größere, sphärische, fluoreszierende Vesikel zu erkennen (Figure 4B, Anhang B). Interessanterweise konnte auch für albuminmodifizierte Nanopartikel eine stark ausgeprägte Abnahme der zellassoziierten Fluoreszenzintensität um 36% (30 min) bzw. 63% (60 min) detektiert werden. Die Ergebnisse deuten darauf hin, dass sowohl spezifisch als auch unspezifisch gebundene proteinmodifizierte Polymerteilchen von Caco-2 Zellen internalisiert werden. Ausgehend von den in der Literatur beschriebenen Eigenschaften der eingesetzten Proteine HSA und WGA ist jedoch nur bei letzterem eine

rezeptorvermittelte Stimulierung der Endocytoserate zu erwarten. Die ausgeprägte Abnahme der zellassoziierten Fluoreszenzintensität im Fall von albuminmodifizierten Nanopartikeln könnte auf erhöhter adsorptiver Endocytose beruhen. Das limitierte Auflösungsvermögen der eingesetzten fluoreszenzbasierten Methode erlaubt jedoch keine näheren Aussagen über den genauen Aufnahmemechanismus von HSA- bzw. WGA-BOD-NP in Caco-2 Zellmonolayer. Um dieser Fragestellung nachzugehen, müssten erweiterte fluoreszenzmikroskopische Methoden oder aber alternative Analysetechniken wie die Transmissionselektronenmikroskopie verwendet werden.

In der vorliegenden Arbeit konnte gezeigt werden, dass WGA-modifizierte Nanopartikel in einem höheren Maß an Caco-2 Zellmonolayer adhärieren als albuminmodifizierte PLGA-NP. Im Vergleich zu Studien mit Caco-2 Einzelzellen war der Einfluss der Konjugation mit Weizenlektin auf die Cytoadhäsivität von BOD-NP jedoch deutlich geringer. Zu klären bleibt, inwieweit diese Ergebnisse auf das stationäre Versuchs-Setup von Mikrotiterplattenassays sowie auf die morphologische und funktionelle Differenzierung der Caco-2 Zellen im Monolayer zurückzuführen sind. In diesem Zusammenhang könnte eine sukzessive Optimierung der Versuchsbedingungen und Erweiterung der fluoreszenzbasierten Analytik auf Ultrastrukturtechniken Unklarheiten beseitigen und zur Aufklärung des Internalisationsmechanismus proteinmodifizierter Nanopartikel beitragen.

ANHANG

Anhang A

Publikation

"Bionanoprobes to Study Particle-Cell Interactions"

C. Fillafer, D.S. Friedl, A.K. Ilyes, M. Wirth, F. Gabor
Journal of Nanoscience and Nanotechnology, Vol 9, 3239-3245, 2009

Anhang B

Publikation

"Interaction of bioadhesive nanoparticles with an artificial intestinal epithelium"

C. Fillafer, A. K. Ilyes, M. Wirth, F. Gabor, wird eingereicht bei Scientia Pharmaceutica

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Bionanoprobes to Study Particle-Cell Interactions

Christian Fillafer, Daniela S. Friedl, Adina K. Ilyes,
Michael Wirth, and Franz Gabor*

Department of Pharmaceutical Technology and Biopharmaceutics, Faculty of Life Sciences, University of Vienna, Althanstraße 14, A-1090 Vienna, Austria

A variety of research reports have provided evidence that the interplay between nanoparticles and biological systems is strongly dependent on the composition as well as on the size of the particles. However, irrespective of that a fine tuning of the interaction characteristics can be attained by functionalisation of the particle surface. In order to be able to monitor such interactions, an analytically accessible model system for potentially target-specific therapeutically relevant nanoparticles (NP) was generated by encapsulation of the highly hydrophobic fluorophore BODIPY® 493/503 into poly(D,L-lactide-*co*-glycolide) (PLGA) nanodroplets. Analyses of the mean particle size and zeta potential revealed that plain and human serum albumin (HSA) conjugated NP can be stored without agglomeration for at least 28 days at 4 °C as well as −80 °C. Although transfer of the particles into commonly used buffers and cell culture medium did not affect the system's stability, protein-containing dispersants should not be used for experiments demanding high sensitivity as distinct quenching effects were observed. Concerning liberation of the fluorescent marker, no release occurred when incubating the suspension for 7 days at 4 °C, while an onset of release was expectedly detected after 3 h at 37 °C. These experimental limitations were taken into account for the particle-cell interaction studies which, as a consequence of the more hydrophilic particle surface, showed an enhanced adhesion of HSA-conjugated colloids to Caco-2 cells by means of flow cytometry and fluorescence microscopy.

Keywords: Nanoparticle, BODIPY, Albumin, Stability, Fluorescence.

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1. INTRODUCTION

The interaction characteristics between particles in the nanometer range and biological systems have been increasingly investigated issues of interest.[1,2] Intensive research in this field partly results from the possible toxicological consequences inflicted with nanoscaled materials,[3] but mainly has been driven forward in order to exploit the promising features of submicron particles for diagnostic and drug delivery purposes.[4] At this, the suitability of a specific nanoparticulate system for therapeutic applications is highly influenced by several key characteristics of the colloids, most important the actual size, core and surface composition. While the core material is rather limited to a range of biocompatible and biodegradable substances, a high number of potential bioactive targeting moieties can be introduced to the particle surface in order to alter the biodistribution profile of the nanomedicine.[5] Consequently, analytical platforms for the study of targeted nanoparticle interactions with biological systems need to be highly sensitive probes and have to consist of pharmaceutically approved materials which offer the possibility of surface functionalisation. In the process of tracking the association and distribution of colloids at the cellular and tissue level, efficient analytical access poses another crucial parameter. At this, fluorescent marking has been widely preferred due to the comparably simple handling of the substances and the qualitative as well as quantitative information obtained from these experiments.[6] Generally, the marker substance is either covalently conjugated with the polymer forming the particle matrix or physicochemically encapsulated into the colloids by appropriate preparation techniques. Ongoing from the latter approach and considering the previously discussed prerequisites, the aim of this study was to design a highly sensitive analytical bionanoprobe. This was practically attained by incorporation of a 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene (BOD; BODIPY® 493/503) in biocompatible as well as biodegradable poly(D,L-lactide-*co*-glycolide) (PLGA) nanoparticles via an oil-in-water (o/w) solvent evaporation procedure.

*Author to whom correspondence should be addressed.

The main objectives for the experimental part of the present study encompassed a detailed characterisation of the colloids' stability in various media under commonly used storage conditions as well as leakage of fluorescent marker from the nanoparticles in order to evaluate the suitability of the system for routine analytical purposes. To exemplarily investigate the potential of BOD-nanoparticles (BOD-NP) as a tool to study the nanoparticle-cell interaction, HSA-conjugated colloids were prepared and compared to plain BOD-NP regarding cytoadhesivity to Caco-2 cells. This epithelial cell line is highly relevant as a point of application for targeted nanomedicines, since it represents an accepted model which can be used to investigate drug delivery via the human intestinal barrier.[7]

2. MATERIALS AND METHODS

2.1. Materials

4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene was obtained from Molecular Probes (Invitrogen Corp., Carlsbad, California, USA). Resomer® RG503H (PLGA; lactide/glycolide ratio 50:50, inherent viscosity 0.32–0.44 dl/g, acid number > 3 mg KOH/g) was bought from Boehringer Ingelheim (Ingelheim, Germany). Human serum albumin, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC), N-hydroxysuccinimide (NHS) and Pluronic® F-68 were purchased from Sigma Aldrich (Vienna, Austria). All other chemicals used were of analytical purity.

2.2. Preparation of BOD-Loaded PLGA-Nanoparticles (BOD-NP)

The fluorescence-labelled PLGA-nanoparticles were produced by an o/w solvent evaporation procedure. Briefly, 1 mg BOD and 400 mg PLGA were dissolved in 2 g ethyl acetate. This solution was emulsified with a 10% aqueous solution of Pluronic by sonication for 50 s yielding the o/w emulsion (sonifier: Bandelin electronic UW 70/HD 70, tip: MS 72/D, Berlin, Germany), which was poured into a 1% aqueous solution of Pluronic. After mechanical stirring (600 rpm) for 1 h at room temperature, the residual ethyl acetate was removed under reduced pressure resulting in the hardening of nanodroplets. The final particle suspension was filtered (1 μm pore size) in order to eliminate aggregates.

2.3. Particle Size and Zeta Potential Analysis

Prior to all measurements the nanoparticle suspensions were diluted 1:20 with double distilled water. The mean particle size and size distribution were determined by dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern Instruments Ltd., United Kingdom). Zeta potential analyses were carried out at 25 °C in disposable folded capillary cells and were performed in triplicate.

2.4. Modification of the Particle Surface

The model protein HSA was conjugated to the nanoparticle surface by a commonly used carbodiimide-mediated coupling procedure.[8] 20 ml of the nanoparticle suspension were purified with 40 ml of 20 mM HEPES/NaOH pH 7.4 (HEPES) on a tangential flow filtration system (Vivaflow 50; 100,000 MWCO PES, Sartorius Vivascience GmbH, Goettingen, Germany) to adjust the pH and to eliminate PLGA monomers as well as non-encapsulated BOD. Subsequently, 240 mg EDAC and 10 mg NHS, each dissolved in 1 ml of the same buffer, were added to the suspension and incubated end-over-end for 2 h at room temperature. To remove excess cross-linking agent, the suspension was washed twice with 40 ml HEPES followed by addition of 1.22 mg HSA in 1 ml HEPES. After end-over-end incubation at room temperature overnight, unreacted coupling sites were saturated by addition of 300 mg glycine in 1 ml HEPES and further incubation at room temperature for 1 h. Finally, the surface modified nanoparticles were washed five times with 20 ml HEPES to remove excess coupling agents and the suspension was concentrated to 15 ml during the last purification step.

2.5. Storage Stability of Nanoparticles

In order to assess the storage stability, plain BOD-NP and HSA-BOD-NP suspended in HEPES were incubated for up to 28 days at 4 °C and −80 °C. After one, seven, fourteen, twenty one and twenty eight days aliquots were drawn from the suspensions stored at 4 °C and subjected to mean particle size-, polydispersity index- and zeta potential analysis. The suspensions stored frozen at −80 °C were thawed after twenty eight days, sonified for 30 s and analysed accordingly.

2.6. Stability of Nanoparticles in Water, Buffers, and Cell Culture Medium

In order to identify suitable dispersants for the particle-cell interaction studies, BOD-NP were transferred to commonly used buffers and cell culture medium. After diluting the BOD-NP suspension 1:20 with isotonic HEPES, aliquots were taken and mixed 1:1 with double distilled water, isotonic HEPES, PBS pH 7.4 (PBS), RPMI 1640 cell culture medium (+10% fetal calf serum) (RPMI + FCS) and a 0.1% as well as 1% solution of HSA in HEPES. The mean particle size and fluorescence intensity of the suspension were determined immediately and after incubation for 90 min at 37 °C.

2.7. Release of Fluorophore

The BOD-NP suspension was diluted 1:20 with PBS pH 7.4 and divided into 1 ml aliquots, which were incubated end-over-end at 4 °C and 37 °C respectively. After 1, 3, 5, 24, 48, 72 and 168 h, 900 μl were transferred into centrifuge tubes and spun down on an ultracentrifuge (100000 rpm/30 min/9 °C; Optima™ MAX Ultracentrifuge, Beckmann Coulter Inc., Fullerton, USA). To quantify the amount of label which leaked from the particles the fluorescence intensity of the supernatant after centrifugation was determined on a microplate reader (ex/em: 480/525 nm; Infinite™ 200, Tecan Group Ltd., Grödig, Austria). Each sample was analysed in triplicate.

2.8. Cell Culture

The Caco-2 cell line was purchased from the American Type Culture Collection (Rockville, MD, USA) and used between passage 28 and 39. Tissue culture reagents were obtained from Sigma (St. Louis, USA) and Gibco Life Technologies Ltd. (Invitrogen Corp., Carlsbad, California, USA). Cells were cultivated in RPMI 1640 cell culture medium containing 10% fetal calf serum, 4 mM L-glutamine and 150 μg/ml gentamycine in a humidified 5% CO_2/95% air atmosphere at 37 °C and subcultured by trypsination.

2.9. Flow Cytometry

The association of plain and HSA-BOD-NP with Caco-2 single cells was investigated using flow cytometry. To grant comparability between the nanoparticle suspensions, dilutions exhibiting the same relative fluorescence intensity were used. These were prepared with isotonic HEPES prior to all experiments and controlled on a microplate reader. For the binding studies 50 μl trypsinated cell-suspension (5×10^6 cells/ml) were mixed with 50 μl of the respective nanoparticle suspension and incubated for 30, 60 and 120 min at 4 °C. Following incubation, unbound and loosely associated particles were removed by centrifugation and washing with 500 μl HEPES (Centrifuge 5804R, Eppendorf; 5 min/1000 rpm/4 °C). The cell pellet was resuspended in 1000 μl PBS-buffer and analysed on an EPICS XL-MCL analytical flow cytometer (Coulter, Miami, USA) using a forward versus side scatter gate for inclusion of the single cell population and exclusion of debris and cell aggregates. Fluorescence was detected at 525 nm (10 nm bandwidth) and the mean channel number of the logarithmic intensities of individual peaks was used for further calculations. Amplification of the fluorescence signals was adjusted to put the autofluorescence signal of unlabelled cells in the first decade of the 4-decade log range. Data of 3000 cells were collected for each measurement.

2.10. Fluorescence Microscopy

In order to visually identify the binding characteristics of plain and surface modified BOD-NP to Caco-2 cells, fluorescence microscopy was performed. Following incubation of 50 μl trypsinated cell suspension (5×10^6 cells/ml) with 50 μl of plain or HSA-BOD-NP for 60 min at 4 °C and washing with HEPES, the Caco-2 single cells were analysed on a Nikon Eclipse 50i microscope (Nikon Corp., Japan) equipped with an EXFO X-Cite 120 fluorescence illumination system. The focus was set to the middle of the cell and the pictures taken were subjected to deconvolution processing by the Image Analyser software.

3. RESULTS AND DISCUSSION

3.1. Characterisation of Plain BOD-NP and HSA-BOD-NP

The labelling of polymeric nanoparticles with fluorescent marker molecules has frequently been employed as a strategy to render colloids trackable and to consecutively enable an investigation of their fate in biological systems.[9] Hereby, mainly two approaches are being pursued. Firstly, a labelled conjugate, which is subsequently used for the production of nanoparticles, can be synthesised by introduction of a fluorescent head group to a polymer backbone.[8–11] This technique offers the advantage of covalent linkage of the dye molecule, which ensures enhanced attachment of the label, but often involves elaborate synthesis, laborious purification and analytically challenging characterisation of the conjugate. Secondly, the fluorophore can be physicochemically entrapped in the polymeric matrix via suitable preparation techniques.[11] At this, the hydrophobicity of the marker molecule has to be selected according to that of the polymer core, since the resulting interactive forces determine stability of the labelling. Exploiting the latter principle, a simple and effective approach for the preparation of fluorescence-marked biodegradable and biocompatible nanoparticles has been developed. The highly hydrophobic compound 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-5-indacene (BOD), which exhibits fluorescein-like spectral properties as well as high photostability and quantum efficiency, was embedded into PLGA cores by a o/w solvent evaporation procedure. This method yielded colloids with a mean particle size of 120.0 ± 1 nm and a narrow size distribution represented by a polydispersity index (PdI) of 0.087. As illustrated by zeta potential measurements, plain BOD-NP exhibited a negative surface charge of -39.2 ± 0.2 mV due to free carboxyl groups of the H-type PLGA present at the particle-dispersant interface. These carboxylate moieties provide potential grafting points for covalent ligand-attachment on the particle surface, which was exemplified by immobilization of the model protein HSA

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via a carbodiimide-mediated active ester method. As a consequence of the successful conjugation with proteinaceous ligand, an increase in mean particle size by about 4 nm was observed concurrent with a decrease of the zeta potential to -30.7 ± 0.8 mV. This reduction of surface potential can be attributed to direct amidation of carboxyl groups as well as shielding of remaining charges by the protein layer. Importantly, despite the surface modification and the slightly lowered zeta potential, the size distribution of HSA-BOD-NP remained narrow throughout the coupling process as indicated by a PdI of 0.092.

3.2. Storage Stability of BOD-NP

In order to grant that a series of particle-cell interaction studies can be performed with the same batch of nanoparticle suspension, sufficient short term storage has to be granted. Consequently, plain and HSA-BOD-NP were continually analysed regarding their mean particle size, PdI and zeta potential as stability parameters, in course of storage over a period of 28 days at 4 °C and −80 °C. As illustrated in Table I, these parameters remained constant for plain and modified nanoparticles incubated at 4 °C. Obviously, despite bearing a layer of immobilised protein molecules and thus slightly lower zeta potential of about −30 mV, the HSA-BOD-NP had not agglomerated after 28 days which is underlined by the low PdI of 0.087. This might be due to a combination of the possibly sufficient electrostatic repulsive forces with steric stabilisation by residual Pluronic from the preparation process.[12] Although storage for four weeks at −80 °C involved freezing and thawing of the colloids, the suspensions exhibited similar stability as compared to incubation at 4 °C. This is illustrated by consistently constant mean particle sizes of 123 ± 1 nm and 126 ± 1 nm for plain- and HSA-BOD-NP respectively, which underline that plain and surface modified suspensions stored at 4 °C or −80 °C can serve as stable reservoirs for comparable long-term studies.

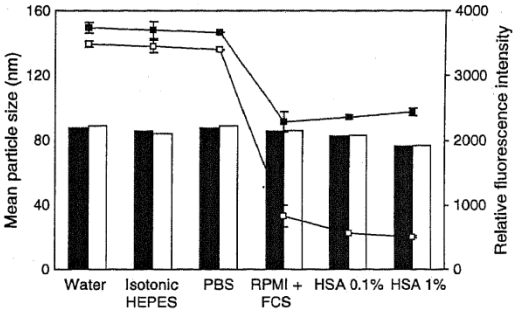

Fig. 1. Mean particle size (bar) and fluorescence intensity (line) of plain BOD-NP in selected dispersants at room temperature (closed) and after 90 min at 37 °C (open).

3.3. Stability of BOD-NP in Water, Buffers, and Cell Culture Medium

Generally, the dispersant's properties like ionic strength, pH-value, and presence of charged macromolecules have to be optimized for the preparation procedure and further storage in order to efficiently prevent the formation of aggregates.[13] However, these suspension media in most cases lack physiological pH conditions as well as isotonicity and therefore do not comply with cells or tissues. Thus, transfer into a cell-friendly dispersant is often deemed to be necessary. To meet this issue, destabilising tendencies and possible effects on the fluorescence intensity of BOD-NP were assessed after dilution of a stock suspension in selected, commonly used media for cell and tissue culture studies.

As illustrated in Figure 1, the mean particle size of BOD-NP remained rather constant, immediately upon

Table I. Stability parameters of plain and modified BOD-NP stored in HEPES at 4 °C/−80 °C.

Storage conditions				
Temperature (°C)	Time	Mean particle size (nm)	Polydispersity index	Zeta potential (mV)
		PLAIN BOD-NP		
4	After preparation	120.0±1.0	0.087	−39.2±0.2
	7 days	121.3±0.6	0.091	−37.2±0.2
	14 days	120.7±0.6	0.081	−37.3±0.8
	21 days	122.0±1.0	0.096	−36.6±0.9
	28 days	122.0±1.0	0.079	−36.5±0.3
−80	28 days	123.0±0.0	0.104	−37.4±0.4
		HSA-BOD-NP		
4	After preparation	124.0±1.2	0.092	−30.7±0.8
	7 days	125.7±1.2	0.099	−31.4±0.5
	14 days	124.7±1.2	0.106	−30.7±0.8
	21 days	125.5±0.8	0.095	−29.9±0.5
	28 days	126.0±1.0	0.087	−30.5±0.8
−80	28 days	126.0±1.0	0.118	−31.5±0.3

mixing and after further incubation for 90 min at 37 °C, with all buffers and media used. Interestingly, the higher concentration of HSA in the dispersant medium led to a slight reduction of the mean particle size as determined by DLS. This effect is very likely due to the presence of large amounts of protein in the medium bearing relatively high diffusion coefficients. Since the DLS-system calculates the mean particle size via the average diffusion coefficient determined in course of the measurement, the size of the BOD-NP is only apparently reduced by the presence of rapidly diffusing proteins, but actually expected to be the same as in water. Regarding the fluorescence intensity of the nanoparticle suspension, rather drastic effects were observed in dispersants containing proteins. While diluting the particles with isotonic HEPES and PBS did not lead to an alteration of the suspension's mean fluorescence intensity as compared to water, the presence of proteins obviously resulted in highly reduced quantum yields. This is explicated by an average reduction of the mean fluorescence intensity by 36% immediately after mixing, which is even increased to 81% after incubation for 90 min at 37 °C. Since similar quenching effects have been reported for BOD dyes covalently conjugated to proteins,[14] the clear drop in fluorescence intensity is expected to be due to physical contact between fluorophore and protein. From this it is inferred, that immediate adsorption of HSA molecules or serum proteins to the nanoparticle surface results in a rapid drop of the suspension's mean fluorescence intensity, which is further enhanced by additional adsorption in course of incubation at 37 °C. While posing a distinct disadvantage for studies to be performed in cell culture medium supplemented with serum, this effect has in contrast been shown to be beneficial for the interpretation of cellular uptake studies.[15] Since cytoinvasion via vesicular internalisation is associated with membrane proteins densely enwrapping the colloid, a reduction of the mean cell associated fluorescence can serve as an indicator to discriminate between sole membrane binding and intracellular uptake.

3.4. Release of Fluorescent Marker from BOD-NP

Undesired release of the fluorescent marker from particles due to insufficient attachment and due to the high surface area available for dissolution has posed a main challenge for the characterisation of a colloid's interaction with biological systems.[16] Especially in the case of particle-cell interaction studies the interpretation of the results is hampered by the presence of freely diffusing marker and possibly generated artefacts thereoff. Thus, as a highly important preliminary step, the liberation of fluorescent marker has to be determined under experimental conditions and accordingly, limits for further studies have to be set in order to minimise misinterpretations.

The release of BOD from unmodified BOD-NP was determined in course of incubation for seven days at 4 °C and 37 °C. In order to solely detect released fluorophore and to exclude particle-associated fluorescence, aliquots of the suspension were subjected to ultracentrifugation at each time point of the analysis. According to fluorescence measurements of the supernatant after centrifugation, 4.1 ng/ml of free BOD were detectable at the start point of the incubation, which represents the quantity of fluorophore not encapsulated in course of the solvent evaporation procedure. When stored at 4 °C, this amount remained constant over the whole incubation period pointing at no release of BOD from the PLGA-nanospheres (Fig. 2). Upon incubation at 37 °C, similarly low quantities of free marker were detected during the first three hours. Following this time lag, a steady increase of non-encapsulated fluorescence was determined which seemed to reach a plateau after 72 h. This markedly higher liberation of fluorophore from the nanoparticles at 37 °C is supposed to be due to increased swelling of the polymer matrix and thus increased solubility and diffusion of BOD to the dispersant phase. Consequently, these results imply that quantitative as well as qualitative measurements using BOD-NP can be performed for at least seven days at 4 °C and about three hours at 37 °C without major artefacts due to released fluorescent label.

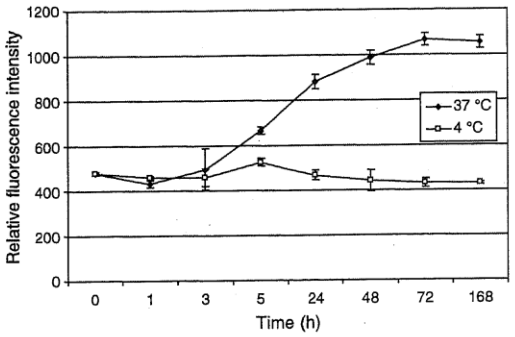

Fig. 2. Release of BOD from nanoparticles in course of incubation at 4 °C and 37 °C.

3.5. Interaction of Plain- and HSA-BOD-NP with Caco-2 Cells

In order to investigate the applicability of plain and surface-conjugated BOD-NP as a model system for a detailed analysis of the nanoparticle-cell interaction, binding studies for 30 to 120 min at 4 °C were performed with Caco-2 single cells using plain and HSA-modified colloids. As the cellular metabolism and fluidity of the membrane is reduced to a minimum at 4 °C, nanoparticles can only associate with the cell surface, but are not actively taken up into the cytosol.[8] Following the incubation and washing of the cells, flow cytometry was used to determine the mean cell associated fluorescence intensity.

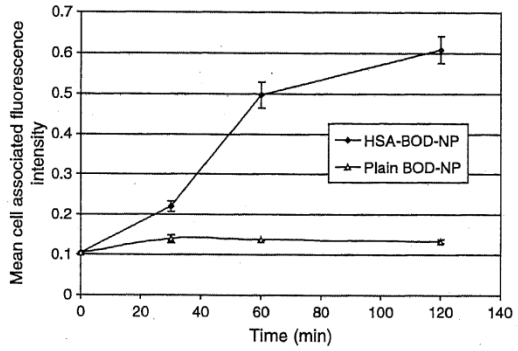

Fig. 3. Mean cell associated fluorescence intensity of Caco-2 cells incubated with plain (-Δ-) and HSA-BOD-NP (-♦-) at 4 °C.

As illustrated in Figure 3, the binding of plain nanoparticles remained constantly low over the whole incubation period and ranged just slightly above the cellular autofluorescene. In contrast, when incubated with HSA-BOD-NP, a distinctly increasing amount of mean cell associated fluorescence intensity was monitored over time. As compared to plain BOD-NP, this difference was reflected by a 58% higher association rate after 30 min. Upon incubation for 120 min, binding of HSA-BOD-NP to Caco-2 cells is even 3.5 times higher, which might be explained by non-specific protein–protein interactions between the HSA-conjugated colloids and the cell membrane, while the low binding in the case of plain BOD-NP is supposed to be a consequence of the poorly cytoadhesive characteristics of the PLGA-polymer. The amount of cell associated plain- and HSA-BOD-NP after incubation for 60 min at 4 °C was also investigated by fluorescence microscopy. As is clearly illustrated by the green fluorescent dot-like structures in Figure 4(A), only few unmodified nanoparticles had associated with the cell membrane. In contrast, a denser corona was visualised for HSA-BOD-NP under the same experimental conditions (Fig. 4(B)) which confirmed the higher cell-binding potential of albumin modified particles as detected in the flow cytometric experiments. These results are especially interesting since HSA is generally known as a model protein mediating only low, non-specific interactions which thus further underlines the potential of BOD-NP as probes to detect slight alterations of the interactive forces between particle and target cell.

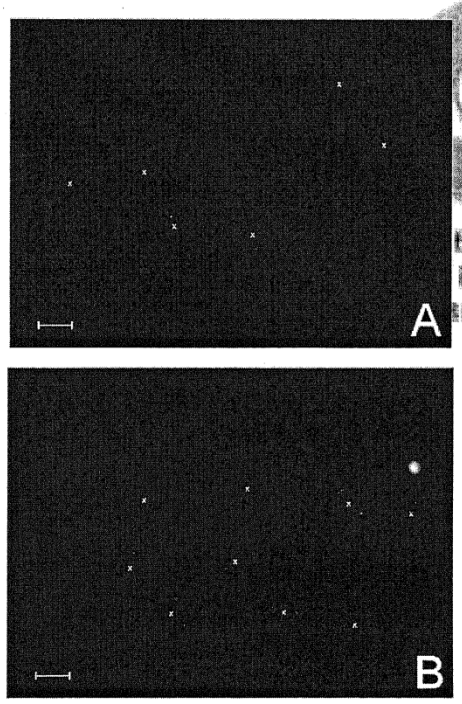

Fig. 4. Caco-2 cells incubated with plain (A) and HSA-BOD-NP (B) for 60 min at 4 °C. The centre of the cell is marked with an "x" for orientation. Scale bar represents 20 μm.

4. CONCLUSIONS

In this work, a novel method to label biodegradable and biocompatible nanoparticles via encapsulation of the compound 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene in PLGA-nanoparticles is presented. The colloids prepared by a o/w solvent evaporation procedure can readily be conjugated with proteins and were shown to be stable for at least 28 days upon storage at 4 °C and −80 °C. Interestingly, transfer of the BOD-NP into protein-containing media leads to drastic quenching of the suspensions' mean fluorescence intensity and thus should be avoided if high analytical sensitivity is desired. Regarding the release of fluorescent label over time it has to be highlighted, that BOD-NP are to be considered stably-labelled when used at 4 °C and that liberation of the fluorophore is negligible for 3 hours at 37 °C. Most importantly and as revealed by binding studies with Caco-2 cells, BOD-NP can serve as highly sensitive probes to analyse alterations in the cell-binding characteristics of nanoparticles as a result of surface conjugation with targeting molecules. At this, it was exemplarily shown that plain BOD-NP exhibit clearly lower membrane binding than colloids surface-modified with HSA.

In conclusion it can be denoted, that the stability of BOD-NP, combined with the ease of covalent surface immobilisation of bioactive molecules, suggests broad applicability of the system to investigate ligand-dominated nanoparticle-cell interactions. Accordingly, BOD labelled colloids are expected to represent an effective and analytically sensitive tool to study potential biotargeting moieties for site-specific diagnostics and nanoparticulate drug delivery.

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INTERACTION OF BIOADHESIVE NANOPARTICLES WITH AN ARTIFICIAL INTESTINAL EPITHELIUM

Abstract

The shuttling of drug-loaded nanoparticles (NP) over the intestinal epithelium into systemic circulation represents a promising approach for the peroral administration of highly potent but chemically sensitive drugs. However, biocompatible and biodegradable nanoparticles produced from polymers like poly(D,L-lactide-co-glycolide) (PLGA) are characterized by a lack of bioadhesion that limits their binding to and translocation through the epithelium. To enhance the bioadhesion of PLGA-NP, the carbohydrate binding protein wheat germ agglutinin (WGA) was covalently grafted to the surface of fluorescent labeled particles. Using Caco-2 cell monolayers the cytoadhesive and cytoinvasive properties of WGA-PLGA-NP and albumin-modified PLGA-NP as control were investigated by fluorimetry and fluorescence microscopy. Surface modification of PLGA-NP with WGA leads to increased cell binding as illustrated by the adhesion of 13.9 ± 1.3% of these particles compared to 8.5 ± 1.5% of the control after 120 min incubation at 4 °C. Upon raising the incubation temperature, both types of protein-modified particles were taken up into the Caco-2 cell layers by energy consuming transport processes. The uptake mechanism and potential translocation of the cell-bound nanoparticles from the apical to the basolateral side of the cells has to be studied further in order to elucidate the potential of WGA-PLGA-NP for the peroral administration of highly potent drugs.

Keywords

PLGA, nanoparticle, WGA, cytoadhesion, cytoinvasion

1. Introduction

Due to the easy handling, high patient compliance and economic reasonability, peroral application is the preferred route for drug administration. However, new highly potent biotech drugs with sensitive structures like peptides, proteins and DNA are instable in the acidic environment of the stomach and are prone to a loss of activity due to enzymatic degradation.[1] At this, polymeric nanoparticles containing the active agent could protect their cargo in the harsh gastrointestinal environment and might subsequently serve as shuttles for the drug over the epithelial barrier into systemic circulation. However, this approach to drug delivery is rather limited since it is widely accepted that the peroral uptake of nanoparticles is too low to generate therapeutically effective plasma concentrations. Using nanoparticles made from poly (D,L-lactide-co-glycolide) (PLGA), it has been shown that the uptake into rat intestinal epithelium is low and strongly dependent on size.[2] Probably, this is a consequence of the reportedly low cytoadhesion of particles prepared from plain PLGA.[3] Modification of the colloids with bioadhesive ligands could mediate their effective attachment to intestinal cells and might consequently result in an increased absorption rate. In this regard surface modification with carbohydrate binding proteins might represent a highly promising approach for enhancing the binding of particulate drug carriers to enterocytes.[4,5] Dietary lectins such as wheat germ agglutinin (WGA) not only resist degradation in the stomach but also strongly associate with mucus and intestinal epithelial cells. These properties account for their potential as bioadhesive coatings of drug carriers for peroral application. Making use of this concept, it has been shown recently, that conjugation of PLGA nanoparticles with WGA leads to drastically improved cytoadhesion to and internalization by Caco-2 single cells.[3]

In the work presented it was to be investigated, whether surface modification of nanoparticles with WGA also leads to enhanced association with epithelium-like Caco-2 monolayers. Therefore, PLGA nanoparticles were used that either had been conjugated with the lectin or with human serum albumin (HSA) as a negative control. In order to be able to analytically monitor the effect on the particle-cell interaction, the nanoparticles were fluorescence labeled

as described recently.[3] To quantitatively and qualitatively investigate the cytoadhesive and cytoinvasive properties of the colloids, a combination of fluorimetry and fluorescence microscopy was used.

2. Materials and Methods

2.1 Materials

BODYPY® 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene) was purchased from Molecular Probes (Invitrogen Corp., Carlsbad, California, USA). Resomer® RG503H (PLGA; lactide/glycolide ratio 50:50, inherent viscosity 0.32-0.44 dl/g, acid number >3 mg KOH/g) was obtained from Boehringer Ingelheim (Ingelheim, Germany). Wheat germ agglutinin from *Tricitum vulgare* was bought from Vector laboratories (Burlingame, USA). Human serum albumin, 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC), N-hydroxy-succinimide (NHS) and Pluronic® F-68 were purchased from Sigma Aldrich (Vienna, Austria). All other chemicals used were of analytical purity.

2.2 Preparation of BOD-PLGA-nanoparticles (BOD-NP)

BOD-NP were prepared by an oil-in-water (o/w) solvent evaporation technique under protection from light sources.[3] Briefly, 0.25 mg BOD and 400 mg PLGA were dissolved in 2 g ethyl acetate. This solution was emulsified with a 10% aqueous solution of Pluronic by sonication (sonifier: Bandelin electronic UW 70/HD 70, tip: MS 72/D, Berlin, Germany) for 50 s yielding the o/w emulsion, which was poured into a 1% aqueous solution of Pluronic. After mechanical stirring (600 rpm) for 1 h at room temperature, the remaining ethyl acetate was removed under reduced pressure. The resulting nanoparticle suspension was filtered (1 µm pore size) to remove aggregates.

2.3 Particle size and zeta potential analysis

Prior to all measurements the nanoparticle suspensions were diluted 1:20 with double distilled water. The mean particle size and size distribution were determined by dynamic light scattering

on a Zetasizer Nano ZS (Malvern Instruments Ltd., United Kingdom). Zeta potential analyses were carried out in double distilled water or highly diluted buffers with low ionic strength (<0.1 mS/cm) at 25 °C in disposable folded capillary cells using the same instrument. All measurements were performed in triplicate.

2.4 Modification of the particle surface

In order to adjust the pH value and to remove PLGA monomers as well as non-encapsulated BOD, 20 ml of the nanoparticles suspension were washed twice with 40 ml of 20 mM HEPES/NaOH pH 7.4 on a tangential flow filtration system (Vivaflow 50; 100,000 MWCO PES, Sartorius Vivascience GmbH, Goettingen, Germany). Subsequently, 240 mg EDAC and 10 mg NHS, each dissolved in 1 ml of 20 mM HEPES/NaOH pH7.4, were added to 20 ml of the purified BOD-NP suspension and incubated end-over-end for 2 h at room temperature. Prior to addition of 1.22 mg HSA or 0.67 mg WGA in 1 ml 20 mM HEPES/NaOH pH 7.4, the suspension was washed twice with 40ml of the same buffer to eliminate excess cross-linking agents. Upon incubation of the suspension end-over-end over night at room temperature, non-reacted coupling sites were blocked by addition of 300 mg glycine dissolved in 1 ml 20 mM HEPES/NaOH pH 7.4 and further incubation for 1 h at room temperature. Finally, the surface modified nanoparticle suspension was washed thrice with 40 ml 20 mM HEPES/NaOH pH 7.4 and concentrated to 20 ml in course of the last purification step.

2.5 Cell culture

The Caco-2 cell line was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and used between passage 40 and 79. Tissue culture reagents were purchased from Sigma (St. Louis, USA) and Gibco Life Technologies Ltd. (Invitrogen Corp., Carlsbad, California, USA). Cells were cultivated in a humidified 5%

CO_2 / 95% air atmosphere at 37 °C in RPMI 1640 cell culture medium containing 10% fetal calf serum, 4 mM L-glutamine and 150 µg/ml gentamycine and subcultured by trypsination. For cultivation in microplates, 17.000 cells/well were seeded in 96well plates and 136.000 cells/well on glass cover slips in 24well plates.

2.6 Microplate binding studies

Confluent monolayers that had been cultivated for 4, 6, 8 or 11 days were used to investigate the interaction of nanoparticles with Caco-2 cells. After removal of the cell culture medium and one washing step with 20mM HEPES/NaOH pH 7.4, the monolayers were cooled to 4 °C. Subsequently, 100 µl suspension of WGA-BOD-NP or HSA-BOD-NP was added and the cells were incubated for 10, 20, 40, 60 or 120 min at 4 °C. Following two washing steps with 20 mM HEPES/NaOH pH 7.4, the monolayer associated fluorescence was determined on a Tecan Infinite M200i microplate reader (ex/em: 480/525) . In order to investigate if nanoparticles are internalized by Caco-2 monolayers, cells loaded with WGA- and HSA-BOD-NP for 120 min at 4 °C were post-incubated for 30 min and 60 min respectively at 37 °C.

2.7 Fluorescence microscopy

Caco-2 monolayers that had been cultivated on glass cover slips were used for nanoparticle binding studies 5-7 days postseeding. Following removal of the cell culture medium and one washing step with 20 mM HEPES/NaOH pH 7.4, the cells were cooled and incubated with WGA-BOD-NP for 60 min at 4 °C. Subsequently, the monolayer was washed twice with 20 mM HEPES/NaOH pH 7.4 and fixed with a 2% aqueous solution of paraformaldehyde. Upon saturation of reactive sites with ammonium chloride and washing, the monolayers were embedded in a drop of FluorSave™ and let to dry over night. The samples were analysed on a Nikon Eclipse 50i microscope (Nikon Corp., Japan) equipped with an EXFO X-Cite 120 fluorescence illumination system.

3. Results and Discussion

3.1 Surface modification of BOD-NP

The physicocolloidal properties of BOD-NP were characterised prior to and after the conjugation with proteinaceous ligands by measurements of the mean particle size, the polydispersity index (PdI) and the zeta potential (ZP). Directly after the preparation procedure the mean particle size of the colloids was 85.4 ± 0.17 nm with a rather narrow size distribution as illustrated by a PdI of 0.116. At this point, unmodified nanoparticles exhibited a zeta potential of -20.4 mV due to the presence of free carboxylic end groups at the particle surface (Table 1).

Table 1: Mean particle size, PdI and zeta potential of plain BOD-NP, HSA-BOD-NP and WGA-BOD-NP

	mean particle size [nm]	**polydispersity index (PdI)**	**zeta potential [mV]**
plain BOD-NP	85.4 ± 0.17	0.116	-20.4 ± 0.9
HSA-BOD-NP	97.7 ± 0.45	0.127	-13.8 ± 0.4
WGA-BOD-NP	98.2 ± 2.63	0.120	-15.2 ± 1.6

In course of the coupling procedure the carboxyl groups were activated with EDAC/NHS and subsequently reacted with human serum albumin (HSA) or wheat germ agglutinin (WGA). Finally, after blocking the non-reacted active ester groups with glycine, the physicocolloidal parameters of the nanoparticles were monitored again. As shown by an increase of the mean particle size by 12 nm and 13 nm for HSA-BOD-NP and WGA-BOD-NP respectively, the surface modification procedure yielded slightly larger nanoparticles due to the protein coating. As illustrated by the consistently low PDIs of 0.127 and 0.120, these colloids were characterized by a narrow size distribution which indicates that the formation of aggregates in

course of the coupling procedure was negligible. Concurrent with the slight increase in particle size, the zeta potential clearly decreased to -13.8 ± 0.4 mV in case of HSA-BOD-NP and -15.2 ± 1.6 mV in case of WGA-BOD-NP respectively. These shifts are a result of the coupling-inherent depletion of free carboxyl groups on the particle surface.[3] Furthermore, not only the covalently bound but also the non-specifically adsorbed biomolecules lead to the build-up of a protein coat that shields underlying charges. Since electrostatic stabilization is limited in the case of suspensions with zeta potentials below ±30 mV, long term stability might not be granted for the surface modified as well as plain BOD-NP. Partially, this lack of electrostatic stabilization is counteracted by the excess amount of surfactant introduced in course of the preparation procedure. However, for long term studies with the same batch of nanoparticles, storage of the suspension at -80 °C is preferable.

3.2 Interaction of surface modified BOD-NP with Caco-2 monolayers

3.2.1 Cultivation period of the monolayers

Upon reaching confluency, Caco-2 cells differentiate to form a monolayer that morphologically and functionally resembles the intestinal epithelium. In order to investigate potential alterations of the cells' binding capacity depending on the duration of cultivation, interactions studies were performed with confluent Caco-2 monolayers four, six, eight and eleven days post seeding. As illustrated by the rather constant mean cell associated fluorescence intensities in Figure 1, the amount of cell-associated f-WGA is similar for all investigated monolayer ages. In contrast, varying binding rates were observed for the surface modified nanoparticles.

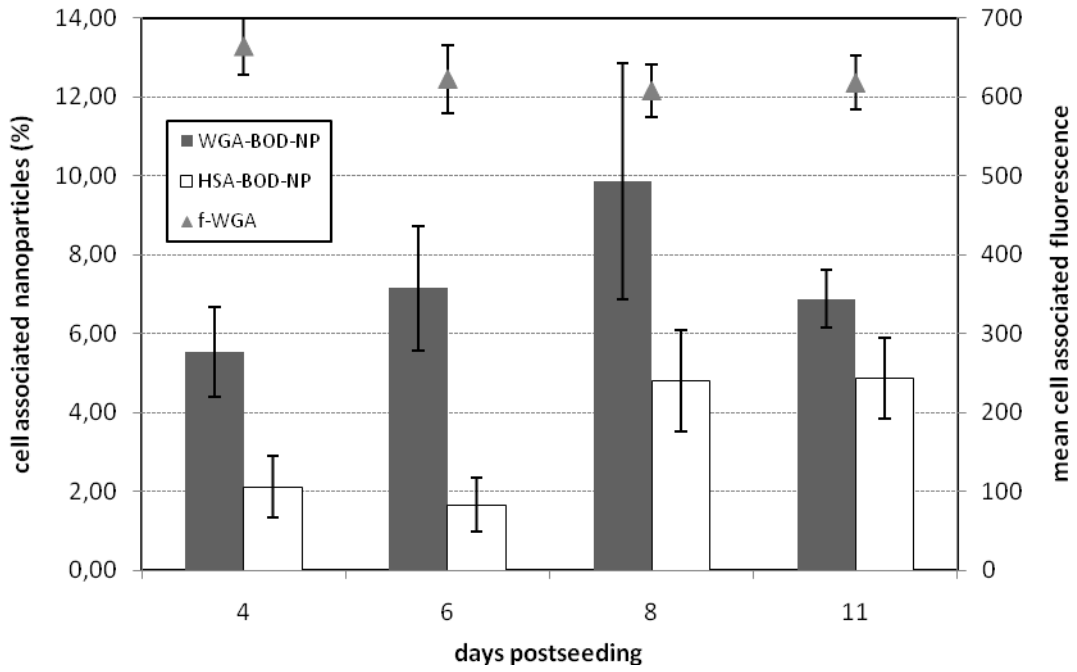

Figure 1: Mean cell associated fluorescence intensity (f-WGA) and percentage of cell associated nanoparticles (WGA-BOD-NP, HSA-BOD-NP) determined by binding studies for 120 min at 4 °C on Caco-2 monolayers 4, 6, 8 and 11 days post seeding.

In the case of WGA-BOD-NP a steady increase in cell-binding was monitored peaking at a monolayer age of eight days. This is illustrated by a shift of the percentage of cell associated particles from 5.53 ± 1.1% to 9.87 ± 3.0% on monolayers which had been cultivated for four and eight days respectively. On day 11 post seeding, the binding capacity for WGA-BOD-NP dropped to a similar level as was observed after six days. Interestingly, different tendencies were monitored for HSA-BOD-NP. While the mean cell associated fluorescence intensity ranged among a constantly low level of 2% in the cases of four and six day old monolayers, an increase to approximately 5% was detected for cultivation periods of eight and 11 days. In terms of the expected differences in cell binding between WGA- and HSA-BOD-NP, the most notable effects were observed with younger monolayers. This is explicated by a 2.6-fold higher association of WGA-BOD-NP on day 4, which even increased to 4.3-fold on day 6. Although the highest amounts of cell associated fluorescence for both types of surface modified particles were detected at day 8, the differences between specific and nonspecific adhesion obviously

decline with prolonged cultivation of the monolayer. As indicated by the constant level of f-WGA binding, these alterations of the nanoparticle-binding are probably not due to differentiation-induced changes of the glycocalyx's carbohydrate composition. Rather than that the morphologic changes occurring in course of cultivation of the cell layer might have contributed to the observed effects. Upon differentiation, the cell membrane's structure is altered by the formation of rod-like microvilli with a length ranging between 0.2 µm and 1.0 µm depending on the cultivation period.[8] This leads to an enlarged cell surface area, which consequently allows for an increased non-specific binding of HSA-BOD-NP (Figure 1). In the case of WGA-BOD-NP, a similar trend is observed, as indicated by an increasing number of cell-associated nanoparticles up to day 8. However, as illustrated by leveling of the bars in case of 11 days post seeding, the non-specific binding of HSA-BOD-NP obviously increased more than the specific binding of WGA-BOD-NP. Possibly, the accessibility of carbohydrate binding sites does not proportionally increase with increasing surface area. With progress of the differentiation process, a relatively small cleft of about 60 nm is established between the microvilli. If the WGA-bindig sites are located near the basis of the microvilli, the diffusion of WGA-conjugated NP to their binding sites might be sterically hindered.[9] Consequently, higher binding rates might be achieved with smaller nanoparticles which are able to diffuse into the clefts.

3.2.2 Time dependency of binding

The time dependent binding profile of HSA- and WGA-BOD-NP to Caco-2 monolayers was monitored over 120 min at 4 °C. As illustrated in Figure 2, the mean cell associated fluorescence intensity due to binding of HSA-BOD-NP or WGA-BOD-NP strongly increased with prolonged incubation periods. In the case of HSA-BOD- NP this is explicated by a clear increase from 3.1 ± 0.5% bound nanoparticles after 10 min to 9.4 ± 0.5% upon incubation for 90 min.

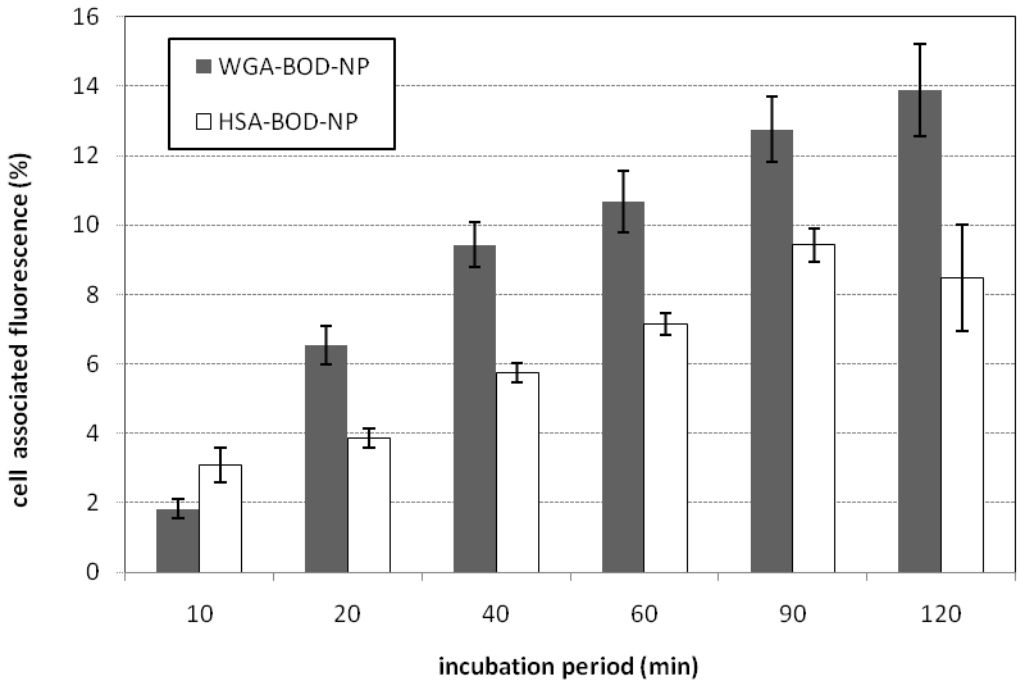

Figure 2: Mean cell associated fluorescence intensity of Caco-2 monolayers incubated with WGA-BOD-NP or HSA-BOD-NP for 120 min at 4 °C.

However, in course of prolonged incubation the mean cell associated fluorescence also seemed to reach a plateau probably indicating saturation. As compared to HSA-BOD-NP the interaction of WGA-BOD-NP with Caco-2 monolayers followed a similar tendency but on a higher level. This is explicated by a 10.9 ± 0.7% difference in binding after 20 min which remained rather constant in course of the whole incubation period. Interestingly, in experiments with Caco-2 single cells, more pronounced differences in cell-binding were observed between WGA-BOD-NP and HSA-BOD-NP. A study has reported 20-fold higher binding of WGA- as compared to HSA-grafted nanoparticles after 300 min incubation while the binding of HSA-BOD-NP remained consistently low in course of the experiment and ranged just slightly above the cellular autofluorescence.[3] In comparison to these results, the difference in cell-binding between WGA-BOD-NP and HSA-BOD-NP on Caco-2 monolayers is rather low. This might be due to several reasons. Firstly, in single cell experiments, the entire cell membrane is available for binding of the analyte. In contrast, in the monolayer setup the

cells are polarized and only the apical side is exposed to the supernatant. If the glycosylated membrane compounds containing N-acetyl-neuraminic-acid and N-acetyl-D-glucosamine are unevenly distributed, this might explain the relative decrease of WGA-BOD-NP binding specificity compared to HSA-conjugated colloids. Secondly, the single cell binding studies were performed under constant agitation. Due to a lack of suitable equipment, this was not possible in the microplate setup. However, effective mixing of the assay, which simulates the dynamic conditions encountered *in vivo*, might lead to a shift in favour of the lectin interaction. Thirdly, after 120 min a saturation of HSA-BOD-NP was observed in contrast to WGA-BOD-NP. Experiments performed for longer incubation periods might reveal more pronounced differences in cell-binding between the lectin- and albumin-modified colloids. However, such studies do not seem reasonable from a biopharmaceutical view, since the constant exposition of nanoparticles to a tissue *in vivo* is not likely to exceed 120 min.

3.2.3 Internalisation of WGA- and HSA-modified nanoparticles

To assess whether the membrane-bound nanoparticles are internalised by Caco-2 cells, a pulse-chase incubation protocol was followed. Upon loading of the monolayers with HSA- and WGA-BOD-NP for 120 min at 4 °C, mean cell associated fluorescence intensities of 92 fluorescence units (FU) and 151 FU respectively were monitored (Figure 3).

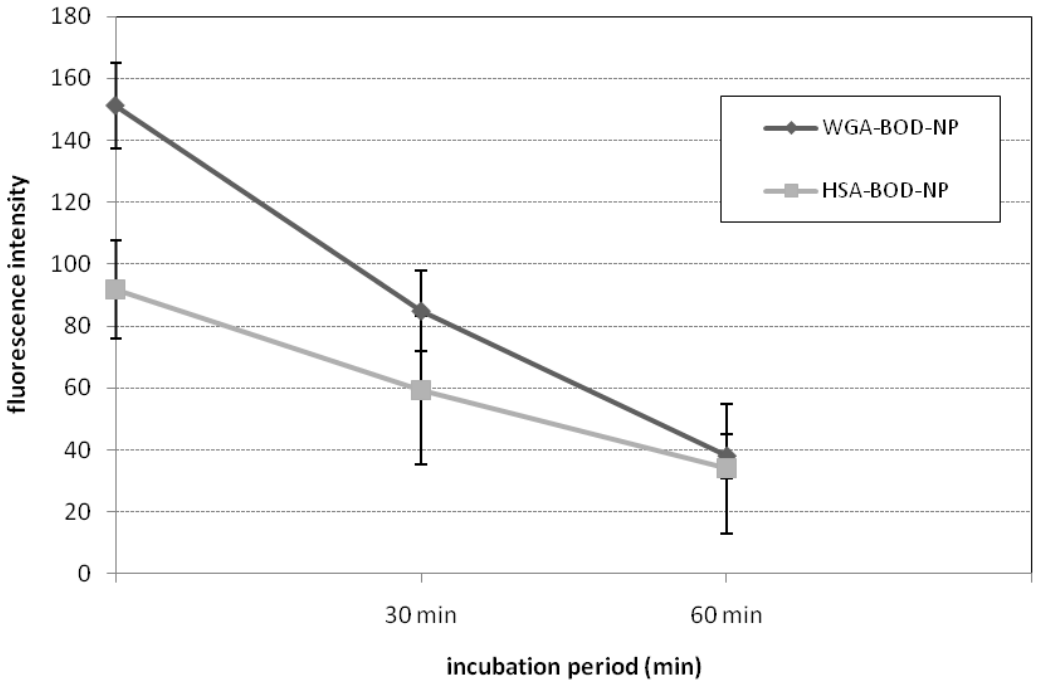

Figure 3: Mean cell associated fluorescence intensity of Caco-2 monolayer loaded with WGA BOD-NP for 120 min at 4 °C in course of further incubation for 60 min at 37 °C.

After washing and raising the temperature level to 37 °C, an incubation period dependent drop of these start levels was detected for HSA- as well as WGA-grafted nanoparticles. In the case of WGA-BOD-NP, the mean cell associated fluorescence intensity experienced a clear decrease by 44% after 30 min and 75% after 60 min respectively. Interestingly, a similar tendency was observed for HSA-modified colloids as indicated by a decrease of the cell associated fluorescence by 36% and 63% respectively. Upon incubation for 60 min at 37 °C, about 40 FU were monitored for HSA- as well as WGA-BOD-NP. These decreases of cell-associated fluorescence intensities indicate the internalisation of specifically as well as non-specifically bound particles into the cell. Taking the higher start level after loading into account, a quantitatively more pronounced internalisation of the glycocalyx-bound WGA-modified colloids can be assumed. The drop of the particles' fluorescence upon the enabling of active transport processes at 37 °C can be explained by internalisation inherent quenching. It has been reported that BODIPY and BOD-NP are susceptible to a loss of fluorescence in the

presence of proteins.[10.11] This mechanism might also be responsible for the decrease of the cell-associated fluorescence intensity upon internalisation in protein-containing vesicles.

3.2.4 Fluorescence microscopy

In addition to fluorimetry, complementary information about the cytoadhesive and cytoinvasive characteristics of WGA-BOD-NP was acquired by fluorescence microscopy. When Caco-2 monolayers were incubated with nanoparticles for 60 min at 4 °C, only binding takes place. This results in a dense pattern of dot-like fluorescent structures on the monolayer as illustrated in Figure 4A. Chase-incubation for 30 min at 37 °C led to the internalisation of WGA-grafted nanoparticles which was characterized by the occurrence of larger, spherical vesicles in the cytosol (Figure 4B).

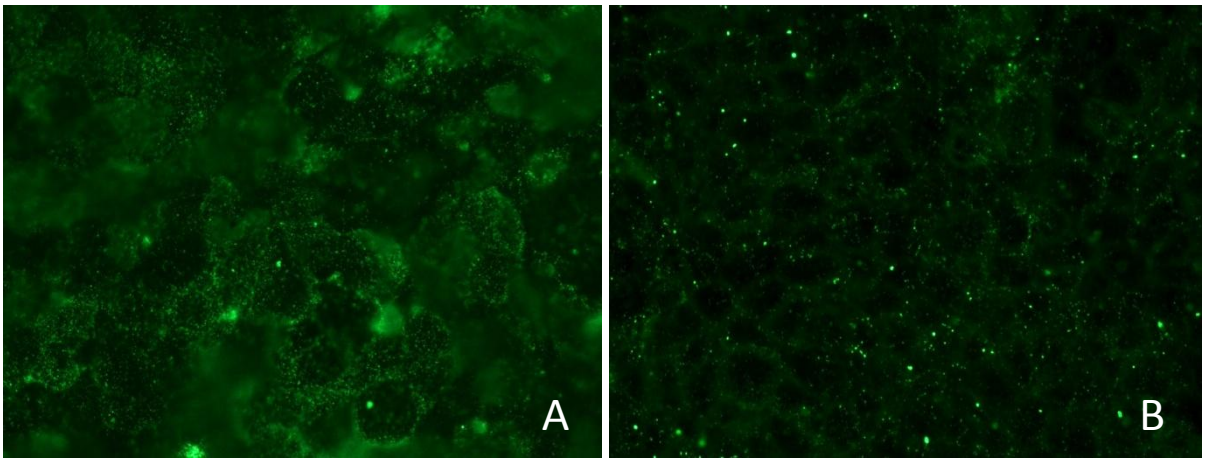

Figure 4: Caco-2 cells incubated with WGA-BOD-NP for 60 min at 4 °C (A) and for further 30 min at 37 °C (B).

4. Conclusions

As illustrated in the work presented, WGA-modified nanoparticles are characterized by a higher binding rate to Caco-2 monolayers than albumin-conjugated colloids. However, the targeting effect due to conjugation with cytoadhesive lectin is not as pronounced as that previously reported in a study using Caco-2 single cells. As shown by the varying binding rates depending on the monolayer age, the moderate targeting effect might be due to the differentiation induced restructuring of the cell membrane in the monolayer. In particular, the formation of microvilli might introduce steric barriers for the diffusion and subsequent association of a lectinized nanoparticle with the glycocalyx. This could be clarified by performing binding studies including differently sized nanoparticles conjugated to WGA. Furthermore, it has to be studied whether conjugation of nanoparticles with WGA leads to an altered intracellular trafficking as compared to non-modified or albumin-modified colloids. Using Ussing chambers or Transwell setups it also remains to be investigated whether internalized particles could be transported to the basolateral side of the intestinal epithelial monolayer. This would clarify if WGA exhibits a transcytosis mediating effect that might be exploited for the shuttling of perorally applied nanoparticles into systemic circulation.

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CURRICULUM VITAE

Persönliche Daten

Name	Adina Katharina ILYÉS
Geburtsdatum, ***-ort***	21.11.1981 in Satu Mare
Staatsbürgerschaft	Österreich
Eltern	Miklos ILYÉS und
	Katharina ILYÉS († 1997)
	Julianne ILYÉS (Stiefmutter)
Geschwister	Szabolcs ILYÉS (1978)

Ausbildung

Allgemeinbildung

1988 - 1989	Volksschule (je ½ Jahr)	Rumänien / Ungarn
1989 - 1993	Volksschule	Gloggnitz
1993 - 1997	Bundesrealgymnasium	Neunkirchen

Kaufmännische Bildung

1997 - 2002	Bundes Handelsakademie	Neunkirchen
	Spezialisiert auf Wirtschaftsinformatik	
6. Juni 2002	**Matura**	

Studium

Oktober 2002	Studium der Pharmazie an der Universität Wien
29. September 2003	Abschluss der 1. Diplomprüfung
Okt. 2007 – Mai 2008	Praktische Arbeit zur vorliegenden Diplomarbeit am Department f. Pharmazeutische Technologie und Biopharmazie
Juni 2009	Abschluss der 2. Diplomprüfung
Juni 2009	Abschluss der 3. Diplomprüfung
September 2009	Diplomprüfung

Studienbegleitende Tätigkeiten

Aug. 2001 - Aug. 2008	Ferialpraktikum in der Stadtapotheke Gloggnitz im Bereich Rezeptur, Warenübernahme und Verkauf
Juli 2002 - jetzt	Geringfügig angestellt in der Stadtapotheke Gloggnitz
Nov. 2006 - Feb. 2009	Geringfügig angestellt im Facultas Pharmashop
März 2007, März 2008	Standbetreuung bei der Berufs und Studienmesse (BeSt^3)
November 2007	Standbetreuung (Österreichischen Apothekerkammer) bei der Frauen/Männer Gesundheitsmesse
SS 2007	Tutor in den Übungen „*Magistrale Arzneimittelherstellung*“ der Universität Wien
WS 07/08, SS 2008	Tutor in den Übungen „*Grundpraktikum aus pharmazeutischer Technologie*“ der Universität Wien

Weitere Kenntnisse

Skills	Teamfähig Ordentlich Verlässlich
Computerkenntnis	Windows Office Paket, WinLine Programmiersprachen: Visual Basic
Sprachen	Deutsch Ungarisch Englisch (einschließlich Wirtschaftssprache) Französisch (einschließlich Wirtschaftssprache)
Hobbys	Malen, Klavier, Gitarre, Sport