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Thiolated silicone oil: Synthesis, gelling and mucoadhesive properties

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ABSTRACT

The aim of this study was the development of novel thiolated silicone oils and their evaluation with regard to gelling and mucoadhesive properties. A thiol coupling of 220 ± 14 and 127 ± 33 μmol/g polymer for 3-mercaptopropionic acid (MPA)- and cysteine-coupled silicone oil was determined, respectively. The dynamic viscosity of MPA–silicone raised significantly ($p < 0.000001$) after oxidation with iodine to a maximum of 523-fold within 1 h. During tensile studies, MPA–silicone showed both the highest results for total work of adhesion (TWA) and maximum detachment force (MDF) with a 3.8- and 3.4-fold increase, respectively, compared to the control. As far as the residence time on small intestinal mucosa is concerned, both silicone conjugates were detectable in almost the same quantities for up to 8 h with 56.9 ± 3.3 and 47.8 ± 8.9% of the initially applied conjugated silicone oil. Thiolated silicone oils can be regarded superior in comparison to commonly used silicone oils due to a prolonged retention time in the small intestine as site of action. Gelling and mucoadhesive features are advantageous for antiflatulent as well as mucoprotective biomaterials. Thus, these novel thiomers seem promising for an upgrade of currently available products for the treatment of dyspepsia, reflux oesophagitis and even inflammatory bowel diseases such as ulcerative colitis or Crohn's disease.

1. Introduction

Silicone oils are mixtures of polydimethylsiloxanes with dimethicone and simethicone as their most commonly used representatives. The difference between them is, that simethicone additionally contains small amounts of silicone dioxide. Those silicone oils, alone or in combination with other drugs, have been evaluated as efficient and well-tolerated agents for versatile gastrointestinal indications. For instance in the treatment of acute diarrhoea [1,2], premedication for upper endoscopy [3] and anorectal ultrasonography [4], functional dyspepsia [5,6] and reflux oesophagitis [7,8]. As dimethicone and simethicone bare antifoaming properties [9–11], they are supposed to disintegrate excess gastrointestinal gas, thus relieving flatulence and abdominal discomfort. Furthermore, simethicone revealed visceral antinociceptive effects on stress-induced colonic hypersensitivity in rats [12] as well as an inhibition of Helicobacter pylori [13].

One major drawback of the current formulations is, that they have to be applied three or four times a day [6,8], which is not fostering patient's compliance. With a prolonged retention time in the gastrointestinal tract, the dosing frequency can be reduced. This can be achieved with thiolated and thus mucoadhesive polymers, which strongly attach to mucosal membranes [14–16]. So-called thiomers have great potential for versatile pharmaceutical applications with the first product containing thiolated chitosan (Lacrimera® eye drops, Croma-Pharma) already entering the European market this year [17]. This class of biomaterials can be obtained by the covalent attachment of a thiol ligand to the polymeric backbone. As the thiolated derivatives feature free thiol groups on their surface, they can interact with the mucus layer and attach due to the formation of disulphide bonds [18]. Furthermore, thiomers bare in situ gelling properties as they can form inter- and/or intramolecular crosslinked networks via oxidative disulphide bond formation between polymer chains [19–21].

It was therefore the aim of this study to develop thiolated silicone oils and evaluate them regarding gelling and mucoadhesive features. The goal was to achieve a prolonged residence time on mucosal membranes as the gastrointestinal tract is the intended site of action. Such novel biomaterials are supposed to be advantageous in comparison to currently available products as the dosing frequency can be reduced. As outlined above, silicone oils can be attributed with antiflatulent and mucosaprotective features. As a consequence, the treatment of excessive gastrointestinal gas accumulation, reflux oesophagitis and even inflammatory bowel diseases such as ulcerative colitis or Crohn's disease can be regarded as pharmaceutical targets for thiolated silicone oils. For thiomer synthesis, an amino-modified silicone oil with a functional

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group equivalent weight of 4400 and a viscosity of 100 cSt was chosen. The thiolation was achieved via amide bond formation between the primary amino groups of the silicone oil side chain and the carboxylic acid group of two thiol ligands, namely MPA and cysteine. Iodine as oxidizing agent was chosen to enhance the viscoelastic properties of the silicone conjugates to give evidence for a successful thiolation. Furthermore the affinity for small intestinal mucosa was evaluated with regard to a prolonged residence time for thiolated silicone oils.

2. Materials and methods

2.1. Materials

Poly[dimethylsiloxane-co-(3-aminopropyl)methylsiloxane] with a functional group equivalent weight of 4400 Da (silicone oil), N,N′-diisopropylcarbodiimide (DIC), N,N′-dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole hydrate (HOBt), 1,1′-carbonyldiimidazole (CDI), 3-mercaptopropionic acid (MPA), L-cysteine hydrochloride monohydrate (cysteine), 4,4′-dithiodipyridine (DTDP), 2,4,6-trinitrobenzenesulphonic acid solution 5% (w/v) in demineralized water (TNBS), iodine, pyridine, triethylamine and 1-(2-methoxyphenylazo)-2-naphthol (sudan red G) were purchased from Sigma–Aldrich (Steinheim, Germany). All other chemicals, reagents and solvents were received from commercial sources.

2.2. Synthesis of thiolated silicone oil

2.2.1. Carbodiimides

The synthesis with carbodiimides was based on commonly applied methods [22,23], but modified for silicone oils. A solution of 1 mmol (1 equivalent) of silicone oil, 2 equivalents of pyridine and 2 equivalents of DCC or DIC, respectively, in 40 mL DCM was cooled to 0 °C. Afterwards 2 equivalents of acid (MPA or cysteine, respectively) dissolved in 2 mL DMSO was dropwisely added. The solution was stirred for 1 h at 0 °C and for 24 h at room temperature.

2.2.2. Carbodiimides and HOBt

The usage of the active ester HOBt in combination with carbodiimides was based on previously described procedures [24–26] and modified for silicone oils. First, 1 mmol (1 equivalent) of silicone oil and 2 equivalents of triethylamine were dissolved in 40 mL DCM. Then 2 equivalents of acid (MPA or cysteine, respectively) dissolved in 2 mL DMSO and 2 equivalents of HOBt were added. The mixture was cooled to 0 °C and 2 equivalents of DCC or DIC, respectively, were dropwisely added. The solution was stirred for 1 h at 0 °C and for 24 h at room temperature.

2.2.3. CDI

The synthesis was based on a previously described one-pot-method [27], but adapted strongly. In brief, a mixture of 2 mmol of acid (MPA or cysteine, respectively) dissolved in 2 mL DMSO and 2 mmol of CDI in 40 mL DCM was stirred for 3 h at room temperature. Then 1 mmol of silicone oil was dropwisely added and the solution was stirred for 24 h at room temperature.

2.3. Purification of thiolated silicone oils

The modified silicone oil solution was purified via filtration and five washing steps with demineralized water until the pH of the aqueous phase was neutral. The residual solvent was removed under vacuum. The thiolated silicone oil was finally centrifuged two times for 10 min at 13,400 rpm and stored at 4 °C until further use.

2.4. Determination of thiol groups with DTDP

Another method to determine the total amount of thiol groups attached to the silicone oil was based on a procedure with DTDP [28,29] and modified for silicone oil. The reaction medium was prepared by addition of triethylamine (0.1%, v/v) to DCM and stirring for 10 min in ambient air. 1.0 mg of the polymer conjugate was dissolved in 500 μL of the reaction medium. Subsequently, 500 μL of DTDP reagent (0.9 mg in 10 mL of reaction medium) was added. After 5 min the reaction was terminated with 30 μL of neat acetic acid. Aliquots of 200 μL were transferred to a microtitration plate and the absorbance at 360 nm was read against DCM with a microtitration-plate reader (Tecan infinite M200 spectrophotometer, Grödig, Austria). The quantity of bound thiol ligand was calculated using a standard curve obtained by the thiol group determination of a series of solutions containing increasing concentrations of cysteine.

2.5. Degree of modification

The degree of modification was determined by measuring the free amino groups of unmodified and modified silicone oil, using TNBS. The unmodified silicone oil served as 100% control. For the assay, 1.0 mg of each conjugate was dispersed in 500 μL of 0.5% w/v sodium chloride and 1% w/v Tween 80 solution. Then 500 μL of 0.1% TNBS solution was added and the mixture was incubated for 2 h at 37 °C [30]. Aliquots of 200 μL were transferred to a microtitration plate and the absorbance was measured at 450 nm with a microtitration-plate reader (Tecan infinite M200 spectrophotometer, Grödig, Austria). The amount of remaining free amino groups was calculated using a standard curve obtained by a series of solutions containing increasing concentrations of cysteine hydrochloride.

2.6. Rheological measurements

2.6.1. With Iodine as oxidizing agent

The gelling properties of the thiolated silicon oils were determined as previously described by our research group [21]. In this study, iodine was chosen as oxidizing agent. In brief, a plate–plate combination rheometer (Haake Mars Rheometer, 379-0200, Thermo Electron GmbH, Karlsruhe, Germany; Rotor: C35/1°, D = 35 mm) was used to determine the viscoelastic characteristics of the modified and unmodified silicone oils. Firstly, 800 μL of each polymer sample was admixed with 400 μL of 10% w/v iodine solution in ethanol. Following that, the samples were incubated at room temperature for 1, 8, 16 and 24 h. Dynamic oscillatory tests within the linear viscoelasticity range were performed with 1 mL aliquots of the samples in triplicate. Unmodified silicone oil served as control. From oscillating measurements, the parameters obtained thereby were the phase shift angle (δ), the shear stress (τ) and the shear deformation (γ). The elastic modulus (G'), the viscous modulus (G'') and the dynamic viscosity (η^*) were calculated by the equations below (1)–(3):

$$G' = \left(\frac{\tau_{max}}{\gamma_{max}}\right)\cos\delta \quad (1)$$

$$G'' = \left(\frac{\tau_{max}}{\gamma_{max}}\right)\sin\delta \quad (2)$$

$$\eta^* = \left(\frac{G''}{\omega}\right) \quad (3)$$

where ω is the angular frequency. The gap between the two plates was 0.5 mm and the temperature was maintained at 37 ± 0.1 °C. For the oscillatory stress sweep measurements, the shear stress was set at a range of 0.5–500 Pa at a constant frequency of 6.283 rad/s (=1 Hz). For the oscillatory frequency sweep vice versa, the frequency was varied from 0.6283 to 62.83 rad/s to investigate the frequency ten times above as well as below the chosen 6.283 rad/s for the oscillatory stress sweep. Now the shear stress was kept constant with 1.0 Pa. The phase shift or phase angle (δ) is defined by $\delta = \tan^{-1} G''/G'$ and indicates whether a material is solid-like or liquid-like. A gel, for example, is defined in rheological terms where the G' and G'' are frequency independent and $\tan\delta$ is less than 1, in contrast to a liquid-like material where $\tan\delta$ is greater than 1.

2.6.2. Viscoelastic properties under simulated intestinal circumstances and upon storage

To evaluate the viscoelastic properties of unmodified as well as thiolated silicone oil under gastrointestinal circumstances, 1 mL aliquots of silicone oil were incubated with 30 mL of 0.1 M phosphate buffer pH 6.8 at 37 °C. After 8 h, the buffer was removed and rheological measurements according to the oscillatory stress sweep studies (Section 2.6.1) were performed. The phase shift ($\tan\delta$) was analysed to proof a gelation of the silicone oil, as this parameter indicates a sol–gel transition. The same experiment was conducted upon storage for 3 weeks at 4 °C.

2.7. In vitro evaluation of mucoadhesive properties

2.7.1. Tensile studies

Tensile studies were performed using excised porcine intestinal mucosa. The mucosa was cut into pieces of the same size (approximately 4 cm^2) and one piece was glued to a stainless steel flat disc (10 mm in diameter) using a cyanoacrylate adhesive. The disc was hung from a laboratory stand with a nylon thread (15 cm) into a beaker. Another piece of mucosa was fixed at the bottom of the beaker and approximately 100 mg of modified or unmodified silicone oil was dropped on the mucosa. The silicones were applied in pure form, as oils, because this represents the active form, which is intended for intestinal targeting. The beaker was placed on a balance, and then carefully raised by a mobile platform until both mucosa pieces came into contact. The contact was determined when the nylon thread holding the upper mucosa piece started to bend. The beaker was filled with 50 mL of 100 mM phosphate buffer (pH 6.8) and the samples were incubated for 5 min at room temperature. Then the mucosa fixed at the bottom of the beaker was pulled down from the upper mucosa piece on the disc at a rate of 0.1 mm/s by manual turning of a regulating knob provided by the mobile platform. The regulating knob was precisely marked and validated via length measurements to ensure the exact displacement of 0.1 mm at each turn. Data points were collected every second by computer software (SartoCollect V 1.0; Satorius AG, Germany) linked to the balance with integrated interface. The force versus displacement curve was analysed to calculate the maximum detachment force (MDF) and the total work of adhesion (TWA) as the area under the curve in accordance with the trapezoidal rule.

2.7.2. Residence time

The water resistance testing was based on a previous study with pig ear skin [31], but strikingly modified. The residence time for up to 8 h of modified and unmodified silicone oils was determined. For the test, the polymer was stained with sudan red G:1 g of polymer was admixed with 12 mg of dye and incubated on a thermomixer (Thermomixer Comfort, Eppendorf, Hamburg, Germany) at 30 °C for 3 h. Afterwards the mixture was centrifuged (10 min, 13,400 rpm) and the supernatant was further used. Freshly excised porcine intestine was cut into small pieces (approximately 4 cm^2) and fixed on a stainless steel basket (diameter: 2.0 cm; height: 3.5 cm) using a cyanoacrylate adhesive glue. Approximately 50 mg of modified or unmodified stained silicone oil was dropped on each piece of mucosa. As for the tensile studies, all silicones were applied in pure form, as oils, because this represents the active form, which is intended for intestinal targeting. The baskets were placed in a dissolution apparatus according to the European Pharmacopoeia containing 900 mL of 100 mM phosphate buffer pH 6.8 at 37 ± 0.5 °C. The fully immersed cylinders were agitated with 50 rpm. After 0.5, 1, 2, 4 and 8 h mucosa samples in triplicate were taken and incubated for 30 min in DCM. The absorbance was measured at 500 nm with a microtitration plate reader (Tecan infinite M200 spectrophotometer, Grödig, Austria). Mucosa samples with 50 mg of the respective modified or unmodified stained silicone oil were instantly extracted with DCM and served as 100% control.

2.8. Resazurin assay

The potential cytotoxic effect of 0.5% (m/v) non-thiolated silicone oil, silicone–cysteine and silicone–MPA was determined by resazurin assay. Approximately 2.5 × 104 cells per well were seeded to a 24-well plate. The cells were incubated for 14 days in minimum essential medium (MEM) supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS) and penicillin/streptomycin solution (100 units/0.1 mg/L) at 95% humidity and 37 °C in an atmosphere of 5% CO_2. Experiments were performed during cell passages 22–28. Pure MEM and Triton X 100 served as negative and positive control, respectively. As the thiolated samples had a high viscosity, they were mechanically dispersed in the medium. After 1 and 4 h of incubation, samples were removed from the cells and washed with isotonic phosphate buffered saline. Subsequently, 250 μL of resazurin solution (44 μM) in FCS- and penicillin/streptomycin-free MEM was added to each well and incubated for 3 h. The fluorescence of the supernatant was measured after background substraction using an excitation wavelength of 540 nm and an emission wave length of 590 nm. Cell viability rates were calculated according to Eq. (4):

$$\text{Cell viability } [\%] = \frac{A_s}{A_c} \times 100 \quad (4)$$

where A_s is the fluorescence of samples and A_c is the fluorescence measured after treatment of cells with MEM.

2.9. Statistical data analysis

Statistical data analysis was performed using the student *t*-test with $p < 0.05$ as the minimal level of significance. The results are expressed as the means of at least 3 experiments ± SD.

3. Results and discussion

3.1. Synthesis and characterization of thiolated silicone oils

A one-pot synthesis for thiolated silicone oil was developed as depicted in Fig. 1. The thiomers were obtained by the covalent attachment of MPA or cysteine to the silicone oil side chain. The respective amide bonds were formed between the carboxylic acid group of the thiol ligand and the primary amino group of the silicone oil. Three different approaches were evaluated, namely with the carbodiimides DCC and DIC alone or in combination with the active ester HOBt and thirdly with CDI. Firstly, carbodiimides were chosen because of their common use in peptide synthesis. Their by-products can be easily separated from the product due to their

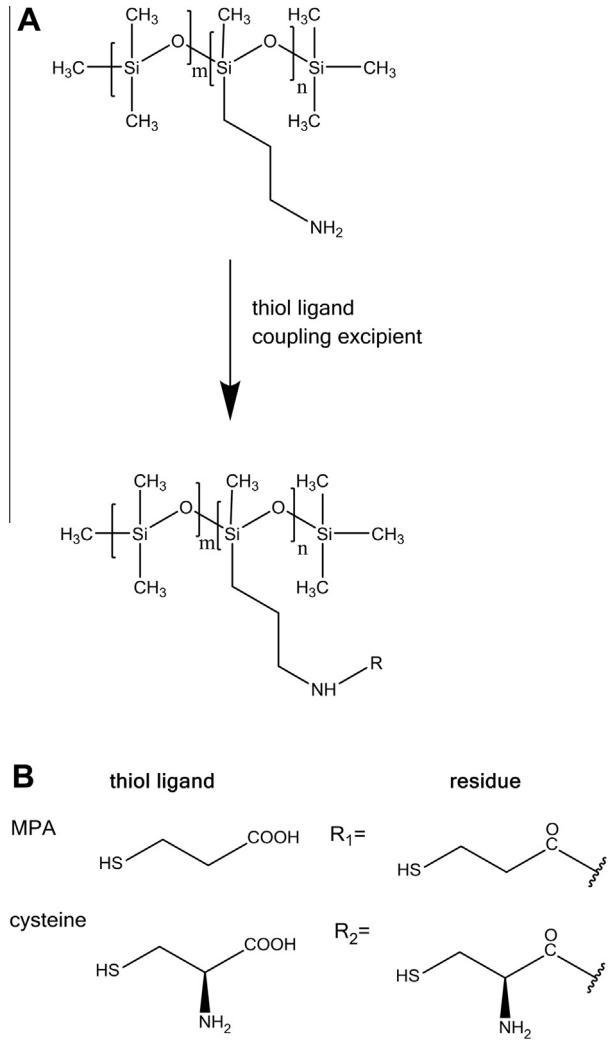

Fig. 1. Scheme of the synthesis: (A) thiolation of amino-modified silicone oil in a one-pot procedure. (B) Applied thiol ligands: MPA and cysteine.

insolubility in most solvents [22]. Secondly, the combination of the coupling reagents DCC and DIC with the active ester HOBt is considered to enhance the reaction rates [22] and reduce the formation of unreactive N-acylurea [23]. The third approach was using CDI to form the amide bond, which could also be managed in a one-pot procedure. The degree of thiolation was determined with DTDP in DCM and for the assay controls were prepared in the same way as the modified silicone oils but without any coupling reagents. Here, the amount of determined thiol groups was negligible indicating only traces of remaining MPA or cysteine (data not shown). For both thiol ligands silicone oil thiomers with raising degrees of free thiol groups could be isolated (Table 1). For MPA-conjugated thiomers, five different products were obtained. For silicone–cysteine, only four modified oils were analysed, as the synthesis with CDI was not successful here. The coupling reagent CDI activates the carboxylic acid via CO_2-formation [27]. For both thiol ligands, only a very slight gas formation during the reaction was visible, which might be an indicator that the activation of the carboxylic acid did not take place efficiently.

The amount of free thiol groups was determined with DTDP. The reason, why DTDP instead of the commonly used 5,5′-dithiobis(2-nitrobenzoic acid), also known as Ellman's reagent, was chosen was the assay medium. As the silicone oils are not water-soluble, only a dispersion of the oils in the aqueous buffer used for Ellman's assay was possible. For DTDP, a procedure with DCM as organic assay medium could be established. So, the silicones could be dissolved and then the thiol groups were analysed. As a comparable Ellman's assay in DCM as organic medium did not lead to reproducible results (data not shown), a thiol group determination with DTDP for silicone oils seems to be more suitable. The results support the assumption that the DIC/HOBt procedure was the most efficient approach with 220 and 127 µmol thiol groups per gram polymer for MPA- and cysteine-conjugates, respectively. As a result, the combination of both carbodiimides with HOBt was more successful than DCC or DIC alone. This can be explained by a reduced formation of unreactive N-acylurea due to the active ester [23]. Sterical hindrance might be an explanation, why DIC is more efficient than DCC for the peptide bond formation. DIC has two isopropyl whereas DCC bares two cyclohexyl residues. Considering the fact, that the provided silicone oil has a functional group equivalent weight of approximately 4400 Da, the theoretical maximum value is around 227 µmol/g polymer. The obtained amount of free thiol groups is in good agreement as no value exceeds this limit. Following that concept, the reaction yield for the two ligands with the DIC/HOBt procedure was almost 100% and slightly below 60% for MPA and cysteine, respectively (Table 1). The results with the highest free thiol group values were gained with the DIC/HOBt synthesis for both MPA and cysteine. Thus this approach seems to be superior in comparison to the other applied procedures regarding the efficiency of silicone oil thiolation.

Table 1
Reaction settings with thiol ligands, coupling reagents, amount of thiol groups determined with DTNB and yield based on a maximum theoretical thiol coupling of 227 µmol/g polymer, respectively (means ± SD).

Thiol ligand	Coupling excipient	SH [µmol/g]	Yield [%]
MPA	DCC	145 ± 38	64 ± 17
MPA	DCC; HOBt	173 ± 6	76 ± 3
MPA	DIC	124 ± 37	54 ± 16
MPA	DIC; HOBt	220 ± 14	97 ± 6
MPA	CDI	109 ± 35	48 ± 16
Cysteine	DCC	83 ± 25	37 ± 11
Cysteine	DCC; HOBt	106 ± 15	47 ± 7
Cysteine	DIC	123 ± 13	54 ± 6
Cysteine	DIC; HOBt	127 ± 33	56 ± 14
Cysteine	CDI	/	/

To further evaluate the efficiency of the coupling reaction, the amount of remaining free amino groups was determined with TNBS. For the calculation, the result for the amino-modified non-thiolated silicone oil was used as 100% control. Accordingly, this value was 201 µmol primary amino groups per gram polymer, which is in quite good approximation to the aforementioned theoretical 100% value of 227 µmol/g amino-modified silicone oil. The assumption made out of the thiol group determination above, that the DIC/HOBt procedure was the most successful for silicone oil thiolation, could be further supported. The lowest yield for free and thus uncoupled amino groups was obtained for the synthesis with DIC in combination with the active ester HOBt with total values of 5% and 21% remaining free amino groups for MPA- and cysteine-conjugated polymer, respectively (Table 2). Considering thiol group characterization, theoretical coupling efficiencies as well as remaining free amino groups, thiolated silicone oils were synthesized in a one-pot procedure using DIC in combination with the active ester HOBt in very high yields. Those thiomers were used for all further studies.

3.2. Gelling properties

3.2.1. Oscillatory stress sweep

Rheological measurements are of the uppermost need as far as mucoadhesive polymers are concerned. It is well known, that a

Table 2
Comparison of remaining free amino groups based on the determination of free amino groups with TNBS (means ± SD).

Thiol ligand	Coupling excipient	NH_2 [μmol/g] determined with TNBS	Remaining free amino groups [%]
MPA	DCC	30 ± 12	15 ± 6
MPA	DCC/HOBt	14 ± 5	7 ± 2
MPA	DIC	35 ± 14	17 ± 7
MPA	DIC/HOBt	10 ± 9	5 ± 4
MPA	CDI	43 ± 17	22 ± 8
Cysteine	DCC	66 ± 7	33 ± 4
Cysteine	DCC/HOBt	52 ± 10	26 ± 5
Cysteine	DIC	50 ± 10	25 ± 5
Cysteine	DIC/HOBt	43 ± 13	21 ± 7
Control	/	201 ± 20	

Table 3
Dynamic viscosity at 37 °C of modified and unmodified silicone oils before and after incubation for 1, 8, 16 and 24 h with 10% w/v iodine solution (means ± SD). Significant difference between control and silicone–cysteine at $p < 0.01$, between silicone–cysteine and silicone–MPA at $p < 0.000001$.

Coupled thiol ligand	Time [h]	Dynamic viscosity [Pa]	Fold-dynamic viscosity
MPA	0	5.95 ± 0.48	81.5
	1	69.20 ± 9.35	523.2
	8	189.79 ± 11.53	78.9
	16	307.48 ± 46.38	47.3
	24	340.79 ± 60.71	38.2
Cysteine	0	0.86 ± 0.10	11.8
	1	1.58 ± 0.43	12.0
	8	6.05 ± 0.89	2.5
	16	17.62 ± 1.88	2.7
	24	17.37 ± 0.90	1.9
Control	0	0.07 ± 0.01	
	1	0.13 ± 0.01	
	8	2.41 ± 0.64	
	16	6.50 ± 1.55	
	24	8.91 ± 1.40	

highly adhesive polymer is quite useless, if there is no inner cohesiveness as otherwise the adhesive bond fails within the polymer itself. A high amount of the polymer will be washed away from the mucosal surface as only a small percentage of the polymer's surface area actually comes into direct contact with the mucosa. An ideal bioadhesive is able to attach to the desired site of action, namely the mucosal surfaces of the gastrointestinal tract, as well as to form a strong inner cohesion. Increasing viscoelastic properties are supposed to proof inter- and intramolecular disulphide crosslinking. Within these studies, free thiol groups covalently attached to the silicone oil side chains were supposed to be oxidized with 10% w/v iodine solution. The dynamic viscosity of thiolated silicone oils at 37 °C was measured after the synthesis as well as after incubation with the oxidizing agent for 1, 8, 16 and 24 h. In order to obtain homogenous mixtures for viscosity measurements, a volume ratio of 1:2 for iodine solution/silicone oil sample was chosen. Unmodified silicone oil served as control. The fact that also the viscosity of the control rises during the experiment might indicate a swelling or congealing effect of the ethanol/iodine incubation medium as such. Apart from that, another explanation might be an oxidation of primary amino groups of the silicone. For example, ketones might be formed, which could form imine bonds with other free silicone oil side chains. Results illustrated in Table 3 however show, that the viscosity of the thiolated conjugates is significantly higher at each time point than the control ($p < 0.01$ for silicone–cysteine and $p < 0.000001$ for silicone–MPA). The longer the mixture of silicone oil and iodine solution was incubated, the more viscous the tested material was. This phenomenon is assumed to be the result of the oxidation of free adjacent thiol groups attached to the silicone oil thus forming disulphide bonds, as has already been described for other thiolated polymers such as thiolated chitosan [21]. A striking increase after 1 h of incubation could be detected as depicted in Table 3 for both thiolated thiomers with a 523- and 12-fold higher viscosity for MPA- and cysteine–silicone, respectively, in comparison to the control. After 24 h a maximum viscosity of 340.8 Pa for MPA- and 17.6 Pa cysteine–silicones was determined. The in situ gelling process for MPA–silicone shows higher viscosities in the beginning as well as during the test compared to the cysteine-conjugate. The reason for this difference is likely to be due to the higher degree of thiolation for the silicone modified with MPA (220 versus 127 μmol thiol groups per gram polymer for MPA- and cysteine–silicone, respectively, see Table 1). The more thiol groups are attached to the polymer, the more inter- and/or intramolecular disulphide bonds can be formed resulting in a higher viscosity of the silicone oil. This correlation has already been shown for other thiomers such as thiolated chitosans [20,32].

The in situ gelling process for MPA–silicone could be confirmed with the loss tangent, $\tan\delta$. If this value is above 1 the substance is regarded as sol. If the loss tangent is below 1, however, the material can be considered as gel. At the beginning of the experiment, all compounds can be regarded as sols with all values exceeding 1 as illustrated in Fig. 2. After 1 and 8 h of incubation with iodine solution, a striking decrease of $\tan\delta$ can be detected for all compounds. For the MPA–silicone conjugate a phase-shift can be detected after 1 h with a loss tangent of 0.7. The MPA–modified silicone stays a gel up to the maximum tested incubation time of 24 h with values for $\tan\delta$ below 1. On the other hand, the results for cysteine–silicone depict a decrease of loss tangent down to 5.8 after 24 h of incubation indicating that it stays a sol throughout the experiment. The results for the thiolated silicone oils are below those for the control with a loss tangent of 49.8 after 24 h for unmodified silicone. In accordance to the above mentioned viscosity measurements, a decrease of loss tangent for unconjugated silicone oil might be explained by a swelling process of the silicone in the ethanol/iodine mixture are an imine bond formation with oxidized amino side chains. Nevertheless, this side reaction has only a minor effect on viscosity compared to disulphide crosslinking, as the thiolated silicone oils show a more pronounced decrease in $\tan\delta$. Within these experiments, the gelling properties of thiolated silicone oils with regard to their behaviour after iodine incubation were evaluated. Both the viscosity and the loss tangent studies support the assumption, that an increase in dynamic viscosity as well as a phase-shift strongly depends on the total amount of free thiol groups covalently attached to the polymer. For the MPA-conjugate with a thiol ligand coupling of 220 μmol/g polymer (Table 1), the greatest augmentation in dynamic viscosity as well as a gelling within 1 h was determined. In contrast, the findings for the cysteine–silicone with less thiol groups (127 μmol/g polymer; Table 1) are less pronounced. Nevertheless, also the cysteine-conjugated silicone oil still shows a substantial increase in the

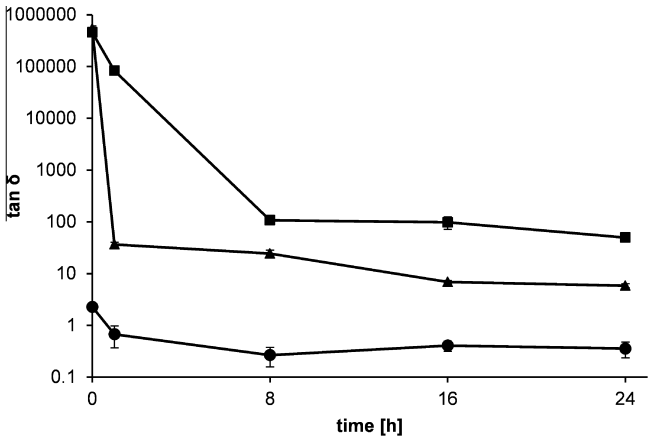

Fig. 2. Effect of 10% w/v iodine solution on the loss tangent determined at 37 °C for MPA (●)- and cysteine (▲)-conjugated as well as unmodified silicone oil (■) as control (means ± SD).

viscoelastic features compared to the non-thiolated control. The assumption that the degree of thiolation is in connection to the viscoelastic properties has already been described previously [20,32], as already outlined above. As rheological studies show, thiolated silicone oils, especially the conjugate with MPA, can be attributed with strong inner cohesiveness. This feature is very important for a sustained delivery of the silicone oil on mucosal membranes as it not only enables the polymer to attach in a thin layer, but also to reside at the site of action in remarkable quantities.

3.2.2. Oscillatory frequency sweep

For this experiment the shear stress was kept constant at 1.00 Pa and the oscillatory frequency (f) was varied from 0.1 to 10 Hz. MPA-conjugated silicone oil was chosen as this was the only compound showing a phase-shift during the incubation with iodine solution. G' is supposed to be a measure of the resistance to elastic deformation as well as a representative of the extent of structuring within the sample [33]. A large G' thus confirms the formation of a strong gel network [34], which in this context is assumed to be due to disulphide bond formation. In the beginning of the experiment, before the formation of disulphide bonds due to an oxidizing agent could take place, the values for G' are low in comparison to the results after iodine incubation (Fig. 3). As the result from the loss tangent measurements above (Fig. 2) indicate, the phase shift for the MPA–silicone already occurs after 1 h of incubation with iodine solution. This gelling process leads to higher G' values for all measured time points after iodine incubation. The most striking raises for G' are on the one hand between 0 and 1 h and on the other hand between 1 and 8 h. This might indicate, that after incubation for 8 h the formation of a strong gel network was mostly finished and thus the major part of free thiol groups has been oxidized. Nevertheless, at every time point, a significant raise of G' was detected ($p < 0.02$). The measurements after 16 and 24 h reveal, except for the first data point, a rather straight line indicating that the frequency of oscillation had no effect on the change of G' (Fig. 3). The findings provide additional evidence for inter- and/or intramolecular crosslinking due to disulphide bonds.

3.2.3. Viscoelastic properties under simulated intestinal circumstances

As the thiolated silicone oils are supposed to show increased viscoelastic properties in the gastrointestinal tract, another study as proof of concept was performed. After incubation for 8 h, the phase shift ($\tan\delta$) was around 0.5 for silicone–MPA, which indicates a sol–gel transition. The cysteine conjugate as well as the unmodified silicone oil showed no gelation with values of around 12 and 150,000, respectively. Apart from that, even upon a storage time of 3 weeks in the fridge, a striking increase of viscoelastic properties for both conjugated silicone oils was evident. Silicone–MPA could be regarded as gel with a phase shift of around 0.6, whereas silicone–cysteine was slightly below 20. Again, the differences between the two derivatives can be explained by a higher amount of free thiol groups for silicone–MPA. The measurements indicate an inter- and/or intramolecular crosslinking of thiolated silicone oil, both under simulated intestinal conditions as well as at low temperatures. So, especially silicone–MPA is a promising and readily gelling silicone oil derivative with an enhanced inner cohesiveness for a prolonged residence time in the gastrointestinal tract.

3.3. In vitro mucoadhesion studies

3.3.1. Tensile studies

This measurement represents a common method for the in vitro evaluation of mucoadhesive strength. The adhesive properties of thiolated silicone oils with regard to intestinal mucosa were measured by quantification of force required to separate two mucosal surfaces with modified or unmodified silicone between them. Such tensile studies can be regarded representative for a small intestinal silicone delivery. The silicone oils are supposed to be active as such in the intestine. A self-emulsifying drug delivery system or mere solid dosage form, such as an enteric-coated capsule, would thus be sufficient. The findings are illustrated in Fig. 4. TWA as well as MDF indicate that the MPA–silicone has an enhanced affinity for the mucosa in comparison to cysteine-modified silicone and control with final values of 154.2 μJ for TWA and 152.4 mN for MDF. The total values and tendencies for both TWA and MDF were in good correlation to each other. The total increase in TWA was 3.8-fold and for MDF 3.4-fold compared to non-thiolated silicone oil. Nonetheless, also for the cysteine-conjugate as substantial augmentation for TWA and MDF could be analysed with a total 2.8- and 2.0-fold increase, respectively. Taking a look at the difference between both thiolated silicones, again the total amount of attached thiol groups might be a likely explanation: MPA-modified silicone with more thiol groups compared to the cysteine-conjugate is able to interact more with the mucosal surface resulting in a greater force for the detachment of the two mucosa pieces. As a result, the higher the amount of free thiol groups attached to the silicone oil, the more pronounced the mucoadhesive features obviously are. Mucus glycoproteins have cysteine-rich subdomains

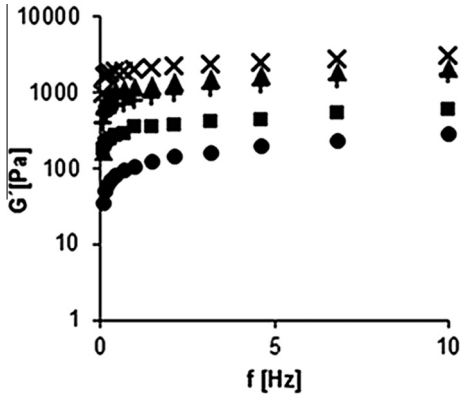

Fig. 3. Elastic modulus G' of MPA-modified silicone oil before (●) and after incubation with 10% w/v iodine solution for 1 (■), 8 (+), 16 (▲) and 24 (×) h (means ± SD). G' is plotted as a function of oscillating frequency. Significant difference between each time point at $p < 0.02$.

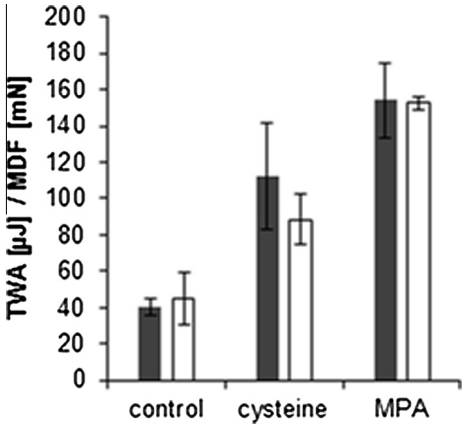

Fig. 4. Comparison of TWA (grey bars) and MDF (white bars) of thiolated silicones compared to the corresponding unmodified silicone as control (means ± SD).

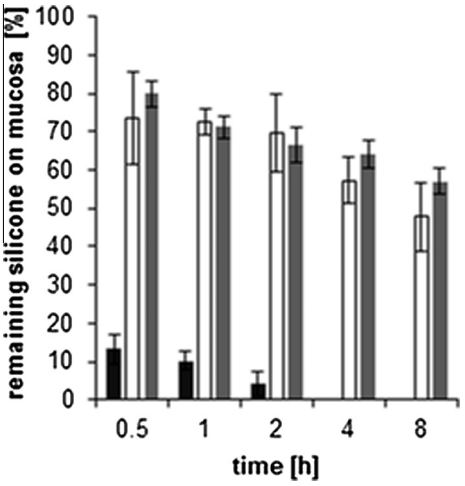

Fig. 5. Comparison of the residence time for MPA – (grey bars) and cysteine – (white bars) modified as well as unmodified silicone oil (black bars) on intestinal mucosa at 37 °C in 100 mM phosphate buffer pH 6.8 after 0.5, 1, 2, 4 and 8 h (means ± SD).

[35,36] featuring free thiol groups. In the human body, those regions dimerize due to a disulphide bond forming. A cohesive network, representing the mucus gel, is formed via this assembly [37]. It has already been shown that also thiolated polymers can interact with free mucus thiol groups and form disulphide bonds [18]. So thiomers seem to be able to imitate this physiological process, which leads to their mucoadhesive properties. A healthy mucus layer plays an important role in the innate defence of the human gut [38] as it protects the gastrointestinal epithelia against mechanical, chemical and pathogen-mediated damage. In addition to that, an advantage in the healing of oesophageal inflammation has already been revealed for dimethicone containing antacid gels in comparison to antacid gels without this silicone oil [7,8]. Taking these facts into account, thiolated silicone oils might be advanced mucosa-protective agents as they strongly attach to mucosal membranes thus providing an additional protective layer.

3.3.2. Residence time

To further evaluate the mucoadhesive properties of thiolated silicone oils, the following study was supposed to imitate the physiological conditions in the small intestine with freshly excised mucosa, 100 mM phosphate buffer pH 6.8 and a gentle stirring. However, the possibility to make predictions for in vivo circumstances is limited, as the silicone oil was dropped on the mucosa before the piece was emerged in simulated intestinal fluid. If the silicone was taken orally, the silicone self-evidently has first contact with intestinal fluids. Nevertheless, enhanced mucoadhesiveness is supposed to directly correlate with residence time on intestinal mucosa. The remaining amount of silicone oil was detected indirectly via sudan red as suitable hydrophobic dye. Results shown in Fig. 5 point out, that unmodified silicone, which served as control, was washed away very fast with only 4.2% remaining after 2 h. After incubation for 4 and 8 h, unmodified control could no longer be determined on the mucosa samples. This is in good agreement to the aforementioned results, as the control has no free thiol groups and is consequently not able to interact with the mucus on the mucosa. In contrast to that, the conjugated silicone oils remain on the small intestine samples for up to 8 h with remaining final amounts of 56.9% and 47.8% for MPA- and cysteine–silicone, respectively. The thiomers can be regarded as silicone oil with spikes allowing them to adhere on the mucosa due to thiol group interplaying as already pointed out above. What is more, the residence time in the small intestine seems to be unaffected by the degree of thiolation. Both modified silicones remain more or less in same quantities on the mucosal membrane, although their total thiol group amounts vary substantially (Table 1). As a result, thiolated silicone oils have a significantly prolonged retention time on small intestinal mucosa in comparison to the unmodified control. Taking these findings into account, another evidence for the assumption, that the thiolation process enhances the mucoadhesive properties of silicone oil is provided. As already outlined above, silicone oil thiomers might be promising mucosa-protective agents with a prolonged residence time in the intestine. The mucosal barrier is of utmost importance concerning inflammatory bowel diseases, such as Crohn's disease. However, the interaction mechanism of thiolated polymers compulsorily depends on a mucus layer. So for example, if the mucus layer is completely lost, which is the case for a more active colitis [39], the thiomers will not be advantageous.

3.4. Resazurin assay

To investigate cytotoxic effects of amino-modified as well as thiolated silicone oils, cell viability of a Caco-2 monolayer was determined. The results were above 95% for all samples up to 4 h of incubation (Fig. 6). Thus, neither amino-modified silicone oil nor the thiomers, silicone–MPA and silicone–cysteine, seem to have a toxic effect within a normal small intestinal passage time.

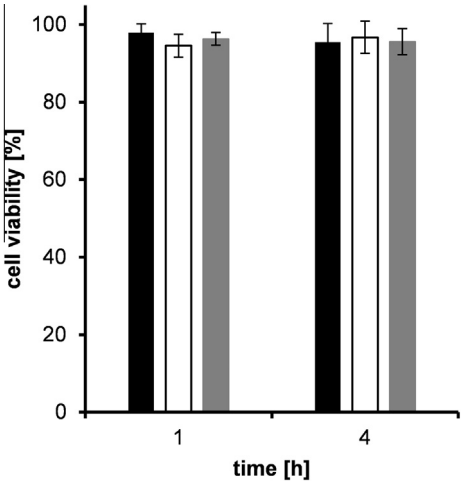

Fig. 6. Cell viability rates of Caco-2 cells after incubation for 1 and 4 h with 0.5% (w/v) MPA – (grey bars) and cysteine – (white bars) modified as well as unmodified silicone oil (black bars) (means ± SD).

4. Conclusion

Within this study, thiolated silicone oils were synthesized for the first time and evaluated as in situ gelling and mucoadhesive polymers. Two silicone conjugates were characterized with different thiol coupling efficiencies. The modified silicone with the higher amount of free thiol groups, namely MPA–silicone, showed the most striking increase in viscoelastic properties with a phase-shift after incubation with an oxidizing agent within 1 h. In addition to that, a striking increase in the dynamic viscosity for MPA–silicone was evident, which was attenuated also the case for the cysteine–silicone with a lower amount of covalently attached thiol groups. As far as the mucoadhesive features of the modified silicones are concerned, the TWA and MDF seem to be in direct correlation with the thiol coupling efficiency as the highest mucosal affinity was determined for MPA–silicone. However, the residence time on mucosal membrane is obviously not affected by the total amount of attached thiol groups with approximately the same results for both thiomers. Both silicone conjugates show a significantly prolonged retention time over 8 h in comparison to the unmodified control. The findings of this study point out, that the thiolation of silicone oil provides new biomaterials with enhanced gelling and mucoadhesive features. It thus opens the door for improved pharmaceutical formulations: silicone oil thiomers might be advantageous in comparison to commonly used silicone oils, such as dimethicone. This agent is supposed to be an antifoaming and mucoprotective agent as it is indicated for several kinds of flatulence as well as heartburn. So, regarding the advanced mucoadhesiveness of thiolated silicone oils, commercially available formulations might be substantially improved due to a prolonged retention time on the mucosal surfaces. As these thiomers are very lipophilic substances, a self-emulsifying drug delivery system, for example, seems to be suitable. Concerning novel therapeutic targets, it might also be feasible to apply thiolated silicone oils in the therapy of damaged mucosal barriers, such inflammatory bowel diseases like colitis ulcerosa or Crohn's disease.

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